

The *mts2*⁺ gene of fission yeast: interactions and mutation analysis

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“A journey is like a person in itself.....The certain way to be wrong is to think that you control it. I feel better now, having said this, although only those who have experienced it will understand it.”

John Steinbeck- “Travels with Charley” 1962

“If you cut too many corners, you go round in circles.”

Anon. 1997

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Abstract

The *mts2-1* mutant of the fission yeast *Schizosaccharomyces pombe* (*S.pombe*) was isolated during a genetic screen to look for cells which were both resistant to the microtubule destabilising drug methylbenzyl-2-carbamylate (MBC) and temperature sensitive (*ts*) (Gordon *et al.*, 1993). The product of the *mts2⁺* gene, Mts2p, is homologous to a growing family of ATPases, known as the AAA family. Mts2p was subsequently shown to be a component of a large multi-subunit complex known as the 26S protease. This complex is responsible for the degradation of proteins which, with few exceptions, have been marked by the covalent addition of polyubiquitin adducts. The targets of this complex include misfolded or damaged proteins, and short-lived proteins such as cyclins and transcriptional regulators. In addition, in mammalian cells, the 20S catalytic core of this complex has been shown to be involved in the production of antigenic peptides which are then displayed on the surface of T-cells.

The primary objective of this work was the isolation and characterisation of gene products which interacted with Mts2p. This was achieved by the use of the yeast '2-hybrid' screen, and enabled isolation of a previously identified *S.pombe* gene *let1⁺*, which is homologous to a gene from the budding yeast *S.cerevisiae*. The product of this homologue, *SUG1*, also a member of the AAA family, was thought to be in transcription or transcriptional regulation. Characterisation of the phenotype resulting from a disruption of *let1⁺* suggested that like *mts2⁺*, it encoded a subunit of the 26S proteasome.

In addition to *let1⁺*, a novel *S.pombe* gene *aps1⁺* was isolated. *Aps1⁺* encodes the *S.pombe* homologue of the mouse MSS1 gene, which was originally isolated as a suppressor of a mutation in a yeast gene encoding a protein kinase. In this laboratory, however, MSS1 was isolated as a multicopy suppressor of the *ts* phenotype of *mts2-1* (Gordon *et al.*, 1993). The interactions between Mts2p, Let1p and Aps1p, the products of *mts2⁺*, *let1⁺* and *aps1⁺* respectively, were studied using the yeast 2-hybrid system. The regions of interaction between Let1p and Mts2p were defined, and the results suggest

that, as is the case for two other ATPase subunits of the 26S proteasome, the N-terminus of each protein is important in mediating this interaction.

In a separate screen to look for genes which were involved in the enhancement of position effect variegation at the centromere, 4 cold sensitive (*cs*) alleles of *mts2* were isolated (J-P. Javerzat, pers. comm.). Mutation analysis was performed on these and on the three *ts* alleles of *mts2*, which had been isolated in the original drug resistance screen (Gordon *et al.*, 1993). All of the mutations lie in a 230 amino acid region which is highly conserved between all members of the AAA protein family (Confalonieri and Duguet, 1995). The phenotype of all of these mutants was studied with respect to morphology, DNA content and MBC resistance. The results indicate that the three *ts* alleles are more drug resistant and have a greater morphological deformation than the *cs* alleles. The implications of this are discussed.

Abbreviations

All units used in this thesis are Standard International (SI) units.

A	adenine
$\alpha\alpha$	amino acids
Abs	absorbance
Ac	acetate
ade	adenine
Apc	anaphase promoting complex
APP	β -amyloid precursor protein gene
Arg	arginine
ARS	autonomously replicating sequence
3-AT	3-amino triazole
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	cytosine
<i>cep</i>	cold sensitive enhancer of position effect variegation
C-terminal	carboxyl-terminal
CDC	cell division control
CDK	cyclin dependant kinase
cDNA	complementary deoxyribonucleic acid
CF-x	conjugate degrading factor-x
CFTR	cystic fibrosis transmembrane conductance regulator
CIP	calf intestinal phosphatase
<i>cs</i>	cold sensitive
DAPI	4, 6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dH ₂ O	distilled water
DMF	dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
dUTP	deoxyuracil triphosphate
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme enzyme

E3	ubiquitin protein ligase
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetra-acetic acid disodium salt
EGTA	ethylene glycol-bis (β aminoethyl ether) N,N,N',N' tetra-acetic acid
EM	electron microscopy
EMM	Edinburgh minimal medium
ER	endoplasmic reticulum
EtOH	ethanol
FACS	fluorescent activated cell sorting
FITC	fluorescein isothiocyanate
G	guanosine
Glu	glutamic acid
Gln	glutamine
Gly	glycine
His	histidine
HMG Co A	hydroxy methyl glutaryl Co-enzyme A
HSP	heat-shock protein
Ile	isoleucine
IPTG	isopropylthio- β -D-galactosidase
kb	kilobase pairs
kDa	kilodaltons
Leu	leucine
Lys	lysine
MBC	methyl benzimidazol-2-yl carbamylate
MCP	multicatalytic proteinase
MCS	multiple cloning site
ME	malt extract
Met	methionine
MgCl ₂	magnesium chloride
min	minute(s)
mRNA	messenger ribonucleic acid
<i>mts</i>	MBC-resistant temperature sensitive
M.Wt	molecular weight
NaOAc	sodium acetate
NLS	nuclear localisation signal
N-terminal	amino-terminal
OD	optical density
PA	proteasome activator

PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pers. comm.	personal communication
PEV	position effect variegation
PFA	paraformaldehyde
Phe	phenylalanine
PI	propidium iodide
PNK	polynucleotide kinase
RNA	ribonucleic acid
rpm	revolutions per minute
rt	room temperature
s	second(s)
<i>S.cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SCS	sister chromatid segregation
SDS	sodium dodecyl sulphate
Ser	serine
SPB	spindle pole body
<i>S.pombe</i>	<i>Schizosaccharomyces pombe</i>
T	thymine
TCA	trichloroacetic acid
TE	Tris: EDTA
Thr	threonine
T _m	melting temperature
tRNA	transfer ribonucleic acid
Trp	tryptophan
Tyr	tyrosine
ts	temperature sensitive
ub	ubiquitin
UBC	ubiquitin-conjugating enzyme
UBP	ubiquitin specific protease
UFD	ubiquitin fusion degradation
UV	ultraviolet
ura	uracil
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
YE	yeast extract

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Chapter 1 INTRODUCTION

1.1 Intracellular proteolysis

The programmed removal of intracellular proteins in prokaryotes and eukaryotes is facilitated by a variety of proteolytic systems. A comparison of the Clp and Lon proteases in eubacteria with the catalytic core of the more complex ubiquitin-dependent proteolytic system in eukaryotes (for reviews see Finley and Chau, 1991; Hershko and Ciechanover, 1992; Luca, 1993), illustrates the structural similarity between these proteolytic complexes in diverse organisms and suggests both a common function and a common origin. Other pathways of protein degradation, such as that which utilises the lysosome or vacuole, may have evolved to fulfil more specialised roles.

The cytosolic degradation mediated by the proteasome is, in most cases, poly-ubiquitin- and ATP-dependent. In the same way, a number of target proteins of the lysosomal degradation pathway, which reach this organelle by endocytosis or by autophagy, are also modified by covalent attachment of ubiquitin. Both lysosomal and non-lysosomal pathways are necessary for cell viability. Whereas regulatory molecules seem to be degraded by a non-lysosomal pathway, bulk protein turnover occurs irrespective of the activity of non-lysosomal pathways (Gronostajski *et al.*, 1985).

1.1.1 Lysosomal protein degradation

Lysosomal protein degradation in mammalian cells, and vacuolar protein degradation in yeast and plants, is a means for the cell to degrade proteins which have been internalised by a receptor-mediated or endocytic pathway. Lysosomes, as well as the ubiquitin pathway, are also responsible for the enhanced protein degradation that results from environmental stress, and that is required for cellular remodelling during differentiation and for removal of cellular components damaged by exposure to toxins. In cultured mammalian cells, 10-30% of long-lived proteins, and thus the bulk of cellular proteins, were thought to be degraded by this pathway (Gronostajski *et al.*,

1985). However, recent work, using inhibitors of the peptidase activity of the 20S and 26S proteasome, has shown that this complex also plays a large part in the turnover of long-lived cellular proteins (Rock *et al.*, 1994). By contrast, inhibition of the proteasome in yeast cells has no effect on the degradation of long-lived proteins by the vacuole, the yeast equivalent of the lysosome, such that the function of this organelle varies in different species (Lee and Goldberg, 1996)

Although lysosomes, which contain enzymes such as acid hydrolases, carboxypeptidases and cathepsins, are the main site of degradation in the endocytic pathway, some degradation also takes place in the late endosomes (Tjelle *et al.*, 1996). Thus, the lysosomes may serve as storage bodies for enzymes which are required during phagocytosis.

A distinct pathway for the lysosomal targeting of cellular proteins such as ribonuclease A (Rnase A) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) require the binding of a pentapeptide targeting sequence KFERQ, within the protein by hsc73, a cytosolic chaperone and a member of the HSP70 family of heat shock proteins (Terlecky and Dice, 1993). The presence of a portion of the hsc73 present within different populations of lysosomes increases the rate of degradation of intracellular proteins degraded by this pathway (Cuervo *et al.*, 1997).

Lysosomes have been implicated in the proteolysis of amyloid beta precursor protein (APP) in Alzheimer's disease (Haass *et al.*, 1992). Intracellular accumulation of this precursor stimulates its degradation by the lysosome. However, this degradation is facilitated by the attachment of ubiquitin to APP (Ali-Khan *et al.*, 1992). The ubiquitination may act as a targeting signal to prevent intracellular accumulation of excess APP.

1.1.2 Organellar proteolysis

1.1.2.1 Proteolysis associated with the endoplasmic reticulum

Initial reports describing the degradation of subunits of the T-cell receptor (TCR), which failed to form into oligomeric complexes, suggested that the degradation took place in a pre-golgi compartment and more specifically in the lumen of the endoplasmic reticulum (ER) (Klausner and Sitia, 1990; Stafford and Bonifacino, 1991; Young *et al.*, 1993). However, recent evidence now suggests that this ER associated degradation is mediated by the association of the ER with the 26S proteasome (Hiller *et al.*, 1996; Hampton *et al.*, 1996).

Degradation of the vacuolar enzyme, carboxypeptidase Y (CBPY), requires the product of the yeast *UBC6* gene, which is associated with the cytoplasmic face of the ER membrane (Sommer and Jentsch, 1993; Hiller *et al.*, 1996). Furthermore, the cystic fibrosis transmembrane conductance regulator (CFTR), which is subjected to extensive post-translational degradation, was found to be degraded by a cytoplasmic ER associated pathway. Degradation of CFTR was prevented by inhibitors of the cytosolic proteasome, demonstrating that the ER itself was not responsible for any proteolysis (Ward *et al.*, 1996; Jensen *et al.*, 1995). Thus it seems that although it is possible that the ER lumen may contain some form of proteolytic system, this remains to be proven.

1.1.2.2 Mitochondrial proteolysis

Genes encoding 3 mitochondrial inner membrane proteases, members of the AAA family of proteins, were isolated in *S.cerevisiae* (Schnall *et al.*, 1994). The proteins encoded by these genes, *YTA10*, *11* and *12*, perform different functions within the mitochondria. The products of *YTA10* and *YTA12* form a complex which, in addition to having proteolytic activity, also acts as a chaperone, and mediates formation of the ATP synthase complex (Arlt *et al.*, 1996; Leonhard *et al.*, 1996). All of these genes are homologous to the bacterial *ftsH* gene, which itself encodes a product with proteolytic activity but no chaperone activity.

1.1.3 ATP-dependent cytoplasmic proteolysis

Although most intracellular proteolysis was known to require ATP (simpson, 1952), identification of the major ATP-dependent proteolytic pathway did not take place until a rabbit reticulocyte lysate, which supported the ATP-dependent degradation of abnormal haemoglobin, was fractionated (Hershko *et al.*, 1979). A small polypeptide, APF-1, which formed covalent bonds with other proteins, was shown to be an essential component of this pathway. This polypeptide was subsequently identified as ubiquitin (Wilkinson *et al.*, 1980). Direct support for the involvement of ubiquitin in ATP-dependent proteolysis was obtained by the identification of the ATP-dependent protease which degraded ubiquitin-lysozyme conjugates, but not free ubiquitin (Hough *et al.*, 1986).

Fractionation of the protease revealed two peaks of activity, one of which sedimented at 26S, and the other at 20S (Hough *et al.*, 1987). Both fractions had protease activity, but only the larger 26S protease, whose activity was stimulated by ATP, was capable of degrading ubiquitin-protein conjugates. The smaller 20S protease, which was thought to comprise 8-10 subunits of a molecular weight between 21-32kDa did not require ATP or ubiquitin for the degradation of protein or peptide substrates. Denaturing gel electrophoresis and limited proteolytic digestion of this 20S complex produced a pattern identical to that produced when the same treatment was applied to the multicatalytic proteinase complex (MCP) (Wilk and Orlowski, 1983). The MCP, a large, multisubunit endopeptidase, was located in the nucleus and cytoplasm of eukaryotic cells, and was initially thought to regulate gene expression by repressing transcription of mRNA (Arrigo *et al.*, 1988). The results of these experiments suggested that the 20S complex was identical to the MCP.

The 26S protease, hereafter referred to as the 26S proteasome is now known to be responsible for the degradation of a wide variety of cellular proteins (Hershko and Ciechanover, 1992). This complex is involved in a diverse set of intracellular processes including the control of the cell cycle, antigen presentation in mammalian cells and the control of gene expression. Since most degradation by this complex requires

the covalent addition of poly-ubiquitin to substrate proteins, this system of modification will now be discussed.

1.2 The ubiquitin pathway for protein degradation

As mentioned above, ubiquitin, an abundant and highly conserved 76 amino acid protein found in all eukaryotic cells, as well as in the archaebacterium *Thermoplasma acidophilum* (Wolf *et al.*, 1993), is an essential component of the major non-lysosomal eukaryotic proteolytic pathway. In *S.cerevisiae*, ubiquitin is expressed from 4 genes, *UBI1-UBI4*. In three of these genes, *UBI1*, *UBI2* and *UBI3*, ubiquitin is synthesised as a fusion to ribosomal subunits (Oskaynak *et al.*, 1987). *UBI1* and *UBI2* encode identical polypeptides fused to the N-terminus of a ribosomal protein of the large complex, whereas *UBI3* encodes a polypeptide fused to a ribosomal protein of the small complex (Finley *et al.*, 1989). The expression of these ribosomal subunits as fusion proteins, from which the ubiquitin must be post-translationally cleaved by the action of a ubiquitin C-terminal hydrolase (see section 1.2.6), increases their incorporation into ribosomes.

By contrast, the *UBI4* gene encodes polyubiquitin arranged as a tandem array of ubiquitin precursors. This is rapidly processed, by the action of C-terminal hydrolases, to monomers (Oskaynak *et al.*, 1987). *UBI4* is expressed in response to stresses such as heat shock, and forms a mechanism whereby proteins which have been damaged by environmental stresses, can be rapidly removed and recycled from the intracellular milieu. Although disruption of the *UBI4* gene in yeast is not lethal, cells become hypersensitive to a variety of stresses. This suggests that whereas the function of *UBI1-UBI3* expression is to maintain a cellular pool of ubiquitin, expression of *UBI4* serves to rapidly increase the concentration of this cellular pool under conditions of stress (Finley *et al.*, 1987).

Only substrate proteins which carry a poly-ubiquitin chain, linked by an isopeptide bond between the C-terminal glycine (Gly⁷⁶) residue of the ubiquitin and a lysine residue in the substrate protein, are efficiently targeted for degradation. The produc-

tion of these chains occurs by the ligation of Gly⁷⁶ to a lysine residue on another ubiquitin molecule. The lysine residue used for attachment varies, and polyubiquitin chains can be formed using lysine at positions 6, 11, 48 and 63 (Baboshina and Haas, 1996). Since Lys⁴⁸ is the major acceptor site for the formation of polyubiquitin chains (van Nocker and Vierstra, 1993), and mutations of Lys⁴⁸-Arg⁴⁸ causes a large decrease in the level of intracellular proteolysis (Finley *et al.*, 1994), formation of polyubiquitin chains through other lysine linkages may reflect the conditions under which the chains are formed, such as environmental stresses. Lys⁶³ linkages are used in the formation of multi-ubiquitin chains for DNA repair, which is promoted by the Rad6p/ Rad18p complex (Spence *et al.*, 1995).

The covalent attachment of ubiquitin to proteins is a multi-step process involving the activity of three types of enzymes, designated E1, E2 and E3 (see Figure 1.1 and below).

1.2.1 Ubiquitin activating enzymes (E1s)

Ubiquitin activating enzymes (E1), catalyse the first step in the transfer of ubiquitin to a protein substrate by creating a high energy thiol ester bond between the free α -carboxyl group of the C-terminal Gly⁷⁶ of the ubiquitin moiety, and a conserved cysteine residue in the E1, in an ATP-dependent step. Three E1 enzymes, encoded by *UBA1*, *UBA2* and *UBA3* exist in budding yeast (McGrath *et al.*, 1991; Dohmen *et al.*, 1995). All of these enzymes have a conserved cysteine residue which is essential for thiol-ester formation. Deletion of the E1 enzymes encoded by *UBA1* and *UBA2* is lethal. However, despite the regions of similarity between the products of *UBA1* and *UBA2* they cannot complement each other's essential functions indicating that these two E1s are functionally distinct (Dohmen *et al.*, 1995). No *in vivo* function has yet been assigned to the product of *UBA3*, and deletion of this gene is not lethal.

In mammalian cells, E1 protein is located both in the nucleus and the cytoplasm. The change in localisation of the enzyme is thought to be dependent on the presence of a

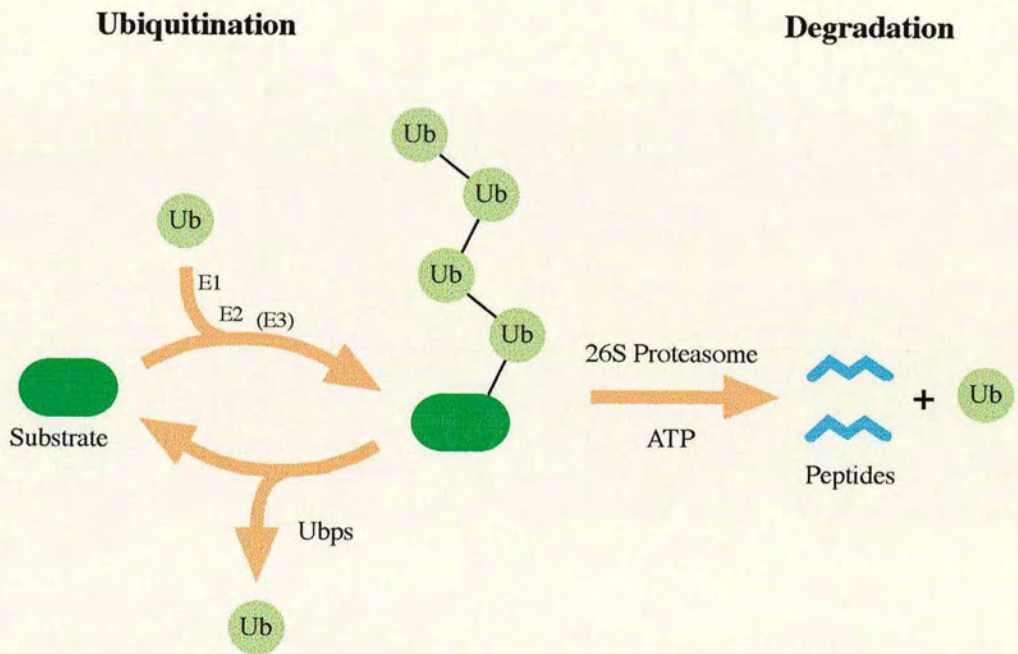


Figure 1.1 Overview of the ubiquitin system for protein degradation (taken from Hochstrasser, 1996).

Isopeptide-linked ubiquitin chains are covalently attached to a substrate molecule by the action of ubiquitinating (E1, E2 and E3) enzymes. This action is opposed by the action of deubiquitinating enzymes (Ubps). Ubiquitinated substrates are degraded by a 2Mda protease called the 26S proteasome.

nuclear localisation signal (NLS) (Kong and Chock, 1992; Grenfell *et al.*, 1994; Handley-Gearhardt *et al.*, 1994). The cytoplasmic pool of E1 is localised to the endosome / lysosome system, the endoplasmic reticulum (ER) and the cytoskeleton. These differences in localisation suggest that ubiquitination may be important in the metabolism of damaged proteins by these organelles, and that it may also be involved in regulating assembly of some aspects of cytoskeleton formation.

1.2.2 Ubiquitin conjugating enzymes (E2s)

The E2 family of ubiquitin conjugating enzymes transfer activated ubiquitin from the E1 to other cellular proteins via formation of an E2-ubiquitin intermediate. All members of this family are structurally related and with a highly conserved 16kDa E2 domain which contains a conserved cysteine residue (Jentsch *et al.*, 1992). E2 enzymes are classified according to the presence or absence of N-terminal or C-terminal extensions of the E2 domain. Class I enzymes possess no extension, whereas Class II E2s have a C-terminal extension and Class III E2s have an N-terminal extension (Jentsch *et al.*, 1990; Matuschewski *et al.*, 1996).

Following sequencing of the *S.cerevisiae* genome, 13 genes, *UBC1-13*, which are predicted to encode E2s, have been identified (Hochstrasser, 1996 and see Table 1.1). Mutations in individual members of this family of enzymes can result in distinct phenotypes, suggesting that the specificity of the ubiquitination reaction may be imparted partly by the number of E2 enzymes.

Overexpression of *UBC1* can partially rescue the phenotype of a *ubc4ubc5* double mutant, despite the fact that the protein encoded by *UBC1* belongs to a different class of enzyme than that of *UBC4* and *UBC5* (Seufert and Jentsch, 1990). This indicates an overlapping function between these enzymes. Ubc2p is identical to the product of the *S.cerevisiae* *RAD6* gene, which, in complex with the product of the *RAD18* gene is involved in DNA repair (Jentsch *et al.*, 1987).

The protein encoded by *UBC3*, is identical to Cdc34p which itself is involved in the ubiquitination and degradation of budding yeast G₁ cyclins, and the cyclin dependent kinase inhibitor, Sic1p. In addition to Ubc3p, several other E2 enzymes have been implicated in the ubiquitination of cyclin in *S.cerevisiae* and in other eukaryotes (Aristarkhov *et al.*, 1996; Yu *et al.*, 1996; Osaka *et al.*, 1997; Townsley *et al.*, 1997). In *S.cerevisiae*, the product of the *UBC9* gene has been implicated in the ubiquitination of the Clb2 and Clb5 cyclins (Seufert *et al.*, 1995). However the failure of the *Xenopus* homologue of this E2 to cofractionate with cyclin ubiquitinating activities in mitotic egg extracts, suggests that another E2 may be responsible for this activity in *S.cerevisiae* (Yu *et al.*, 1996).

In *Xenopus laevis*, two proteins containing mitotic cyclin ubiquitinating activities have been isolated. One of these is the homologue of the *S.cerevisiae* Ubc4 protein. The other E2, termed UBCx, shares a 65% identity at the amino acid level with E2-C, an enzyme that supports mitotic cyclin ubiquitination in extracts of clam oocytes (Aristarkhov *et al.*, 1996; Yu *et al.*, 1996). Both UBCx and Ubc4 can independently catalyse cyclin ubiquitination. However, whereas UBCx converts cyclin into low molecular mass ubiquitin conjugates, UBC4 catalyses the formation of higher mass conjugates (Yu *et al.*, 1996). This difference in 'processivity' may reflect distinct roles for the two enzymes in cyclin B ubiquitination.

E2 enzymes involved in mitotic cyclin ubiquitination have also been isolated from *S.pombe* (Osaka *et al.*, 1997) and from human cells (Townsley *et al.*, 1997). The human enzyme UBC-H10, is 61% identical to the clam E2-C enzyme, and can substitute for E2-C *in vitro*.

The multi-functionality of the E2 enzymes is demonstrated in part by the ubiquitination of the yeast Mat α 2 repressor. This protein is ubiquitinated by at least 2 different pathways, one of which involves the products of *UBC4* and *UBC5*, and the other of which involves the products of *UBC6* and *UBC7* (Chen *et al.*, 1993). These two latter E2 enzymes also have other cellular functions. The product of *UBC6* is associated with the ER, and is involved in the degradation of the vacuolar enzyme carboxypeptidaseY (Hiller *et al.*, 1996). By contrast, the product of *UBC7*, which is also

homologous to *UBC1,4* and 5, is required to mediate tolerance to heavy metals such as cadmium (Jungmann *et al.*, 1993).

The cellular role of a number of members of the E2 family has not yet been elucidated (see Table 1.1). It is likely, however, that their function will partly overlap with that of other E2s. The combinatorial association of these enzymes, as demonstrated by the ubiquitination of Mat α 2, may be responsible for imparting a degree of substrate specificity of ubiquitination. This is also facilitated by the association of E2 enzymes with their cognate E3 ubiquitin protein ligases.

The different phenotypes observed when different E2s are mutated or deleted, suggests that a number of these enzymes have a non-overlapping function. Mutations in the *S.cerevisiae* *RAD6* gene, encoding a Class I E2 enzyme with neither a C-terminal nor N-terminal extension, result in UV sensitivity and defects in sporulation and DNA repair (Jentsch *et al.*, 1987; Spence *et al.*, 1995). By contrast, mutations in the C-terminal tail of Ubc2p (Cdc34p) result in a cell cycle arrest at the G₁-S transition (Goebl *et al.*, 1988). Despite these functional differences, transplantation of the C-terminal tail of Cdc34p onto the conserved region of Rad6p resulted in a chimeric protein which could complement both the cell cycle functions of Cdc34p and the DNA repair and sporulation functions of Rad6p (Kolman *et al.*, 1992; Silver *et al.*, 1992).

1.2.3 Ubiquitin protein ligases (E3s)

E3 enzymes, the ubiquitin protein ligases, act in conjunction with E2s to promote the formation of ubiquitin adducts on target proteins. Although E3s are not always required *in vitro* or *in vivo* for attachment of ubiquitin, at least one member of this family, E6-AP, is known to form a thiolester with ubiquitin, thereby acting as an intermediate in the transfer of ubiquitin from E2 to substrate (Scheffner *et al.*, 1995).

Table 1.1 The *S.cerevisiae* E2 ubiquitin-conjugating enzymes (adapted from Hochstrasser, 1996).

<u>Protein</u>	<u>kDa</u>	<u>Function</u>
Ubc1	24	Important for growth from spores and endocytosis of membrane proteins
Ubc2 / Rad6	20	DNA repair, induced mutagenesis, sporulation, N-end rule pathway
Ubc3 / Cdc34	34	G ₁ -S cell cycle progression, degradation of p40 (Sic1) Gcn4 and Clns
Ubc4 / Ubc5	16	92% identical. Degradation of Mat α 2 and ubiquitin fusion proteins, endocytosis of membrane proteins
Ubc6 / Doa2	28	Degradation of Mat α 2 and some membrane proteins Localises to ER envelope.
Ubc7	18	Resistance to cadmium. Degradation of Mat α 2 and certain membrane proteins. Interacts with Ubc6.
Ubc8	25	Not essential
Ubc9	18	Essential for viability. Required for G ₂ -M cell cycle progression, and degradation of Clb2 and Clb5 cyclins.
Ubc10 / Pas2	21	Peroxisome biogenesis
Ubc11	17	Not determined
Ubc12	21	Not determined
Ubc13	18	Not determined

E2 enzymes involved in mitotic cyclin ubiquitination

UBCx, UBC4	<i>X.laevis</i>
E2-C	<i>Spisula</i>
UBC-H10	<i>H.sapiens</i>
UbcP4	<i>S.pombe</i>

E3 enzymes were originally identified by their ability to stimulate substrate ubiquitination, bind to an E2 and to bind to a substrate (Hershko and Ciechanover, 1992). However, E6-AP did not seem to fulfil all of these criteria. Moreover, prior to the discovery of E6-AP, sequence homology between E3s was limited to the region containing the conserved cysteine residue. Now, however, members of the E3 family belong to one of 2 sub-families, based on a region of homology at the C-terminus of some members to that of E6-AP (Huibregtse *et al.*, 1995)

Two E3s, designated E3 α and E3 β were purified from rabbit reticulocytes (Gonda *et al.*, 1989). One of these, E3 α was found to be the functional homologue of the budding yeast Ubr1 protein (Bartel *et al.*, 1990). Both Ubr1 and E3 α are responsible for the ubiquitination of substrates of the N-end rule pathway (see section 1.2.5). By contrast, E3 β recognises signals which involve residues downstream of the N-terminus.

Until recently, the only other E3 which had been isolated was the mammalian E6 associated protein (E6-AP). This protein, when associated with the human papilloma virus 16 (HPV16) E6 protein, promoted the ubiquitination and subsequent degradation of the human p53 tumour suppressor gene (Huibregtse *et al.*, 1991).

A number of genes encoding potential E3 enzymes have been isolated on the basis of homology to the C-terminus of E6-AP. This *hect* (homologous to E6-AP carboxyl terminus)-domain contains an essential conserved cysteine residue which is utilised in the formation of a thiolester between the ubiquitin and the E3 protein. By contrast to this mode of action, it is thought that members of the second sub-family, comprising the Ubr1-like enzymes, may act as docking proteins for target substrates and for the relevant E2, thereby facilitating the transfer of ubiquitin onto the substrate protein (Varshavsky, 1992).

There are 5 different *hect*-domain proteins in the yeast genome (Hochstrasser 1996). One of these, Rsp5/ Nsi1, is required for the ubiquitination of the yeast uracil permease, an integral plasma membrane protein (Galan *et al.*, 1994). This targeting of a membrane associated substrate may be facilitated by the presence of a Ca²⁺ lipid

binding domain within the polypeptide, a motif shared between other *hect* E3s such as the homologues of Rsp5 in fission yeast, Pub1p (Nefsky and Beach, 1996), and in mouse, Nedd4 (Huibregtse *et al.*, 1997).

Though proteins containing *hect* domains were isolated on the basis of sequence homology, the gene encoding Pub1p was fortuitously isolated in a screen designed to detect novel protein tyrosine kinases in *S.pombe* (Nefsky and Beach, 1996). The homology of the catalytic domain to that of E6-AP, coupled with the isolation of a ubiquitin thiol-ester adduct of Pub1p from fission yeast, showed that Pub1p possessed E3 activity (Nefsky and Beach, 1996). This protein is involved in the ubiquitination of the Cdc25p phosphatase, which itself is required for entry into mitosis.

In addition to the E3s described above, other individual proteins or protein complexes, function as E3s. Ubiquitination of mitotic cyclins in clam extracts requires the APC and an E2, E2-C (Aristarkhov *et al.*, 1996). Though the entire APC has properties of an E3, none of the subunits bears homology to any identified E3 protein. The relevant E2 may also be one of the unidentified proteins within this complex. Several of the proteins in this complex, Cdc16p, Cdc23p and Cdc27p are known to contain the tetra-tricopeptide (TPR) motif (Lamb *et al.*, 1994), which is proposed to mediate protein-protein interactions (Hirano *et al.*, 1988). This may act to bind both the cognate E2 and the ubiquitin moiety.

Other candidates for acting as E3 enzymes include Rad18p, which forms a complex with Rad6p, and is essential for tolerance to UV and ionising radiation in budding yeast (Bailly *et al.*, 1994). Another potential E3 complex may comprise the products of the *CDC4*, *CDC53* and *SKP1* genes. Along with the product of *CDC34*, the products of these three genes interact genetically (Bai *et al.*, 1996; Willems *et al.*, 1996), and are involved in the targeting of the yeast cyclin-dependent kinase inhibitor p40^{siel} for ubiquitin mediated proteolysis during the G₁-S transition. Since Cdc53p associates with the phosphorylated form of the CLN2 G₁ cyclin, but not with an unmodified form, it may be that the product of *CDC53* acts as the substrate recognition component of this complex (Deshaies, 1997).

1.2.4 Specificity of substrate ubiquitination

1.2.4.1 Combinatorial specificity

Since it seems likely that there are only three functional E1 enzymes in yeast, but a larger number of E2 and E3 enzymes, it is tempting to speculate that the combinatorial associations of different E2s with E3s is a major determinant of the specificity of substrate ubiquitination. Such combinatorial specificity has been demonstrated by looking at the interaction of *hect* E3s with different E2s (Huibregeste *et al.*, 1995). This E3 family will accept ubiquitin from the Ubc8 enzyme, but not from Ubc1.

The C-terminal and N-terminal extensions present on the Class II and Class III E2 enzymes respectively, can facilitate the E3-independent ubiquitination of certain substrates such as histones (Kaiser *et al.*, 1994). It is therefore likely that these N- or C-terminal extensions obviate the requirement for an E3 and thus impart the ability of these E2s to interact directly with the substrate.

1.2.5 Cis-acting sequences recognised by the ubiquitin pathway

The addition of poly-ubiquitin adducts onto target proteins requires the recognition of the target protein by the enzymes and co-factors of the ubiquitin system. Without specific recognition, all proteins would be ubiquitinated, thereby targeting many for degradation by the ubiquitin dependent proteolytic system. What are the signals that are recognised by the ubiquitin system? Is it the conformation of a protein, or its phosphorylation state, or is it one or a number of motifs recognisable in the ternary structure of a polypeptide? Although the exact answer to these questions remains a mystery, a number of factors are thought to influence the susceptibility of a protein to ubiquitination.

1.2.5.1 The N-degron

The 'N-End rule' was proposed as a mechanism whereby proteins were targeted for ubiquitin mediated degradation (Varshavsky, 1992). This pathway has been shown to function in *E.coli*, yeast and mammalian cells, and relates the *in vivo* half-life of a protein to the identity of the N-terminal residue. The presence of this pathway, al-

though not a major contributor to proteolysis in eukaryotic cells (Varshavsky, 1996), enables the removal of proteins which have been synthesised without an N-terminal methionine, or which have been processed by an endopeptidase, thereby exposing a destabilising N-terminal residue.

The N-Degron, formed by a combination of the destabilising N-terminal residue with the presence of a spatially important lysine residue, constitutes the recognition motif of the substrate protein (Scheffner *et al.*, 1993). The N-terminal residue is recognised by the E3 Ubr1p, the yeast homologue of the mammalian E3 α , which acts as a docking protein for substrate binding and presumably for binding of the cognate E2, Ubc2.

1.2.5.2 The *S.cerevisiae* Mat α 2 repressor

The yeast Mat α 2 repressor possesses 2 distinct degradation signals which are recognised by different combinations of Ubc proteins, and which are required for its ubiquitin-dependent degradation. *Deg1*, a transplantable N-terminal ubiquitin-dependent degradation signal located within the first 67 amino acid residues of Mat α 2, is targeted by a complex of Ubc6 / Ubc7. By contrast, a complex of Ubc4 / Ubc5 recognises a different signal (Chen *et al.*, 1993). Mat α 2 is stabilised in yeast strains deleted for the above *UBC* genes, though not in a strain deleted for the gene encoding the E3 component of the N-End rule pathway, Ubr1, suggesting that this pathway is not involved in Mat α 2 ubiquitination and degradation (Chen *et al.*, 1993).

1.2.5.3 Destruction boxes

1.2.5.3.1 Cyclin B

The destruction of mitotic cyclins is required for exit from mitosis (Holloway *et al.*, 1993; Surana *et al.*, 1993). In cyclin B from *Xenopus*, 2 regions within the first 90 amino acid residues of the N-terminus have been shown to be required for cell-cycle

mediated activation and degradation. The first of these regions is the D-box, which is located within the first 53 residues of the protein (Glutzer *et al.*, 1991). This 9 amino acid region contains two invariant residues, four highly conserved residues, and one residue that serves to differentiate between A and B type cyclins. Deletion of this region prevented cells from exiting mitosis, causing them to arrest at anaphase (Holloway *et al.*, 1993; Surana *et al.*, 1993). By contrast, the fusion of this N-terminal region onto a foreign protein produces a hybrid protein which becomes susceptible to ubiquitination and proteolysis at mitosis (Glutzer *et al.*, 1991). The second region, which lies between residues 54-90, is lysine rich, and has 4 lysine residues in close proximity to the destruction box. It is likely that following initial recognition of the cyclin molecule, *via* its D-box, this region may be required for the ubiquitination of the cyclin molecule or of any fusion protein carrying this N-terminal region.

1.2.5.3.2 Cut2p

Like cyclin B, the degradation of the fission yeast Cut2 protein occurs during mitosis and requires an N-terminal region which functions like a D-box (Funabiki *et al.*, 1996b). Unlike cyclin B, however, proteolytic degradation of Cut2p is essential for sister-chromatid separation (SCS), and requires the product of the *cut9⁺* gene, a subunit of the APC. Expression of a non-degradable form of Cut2p, which lacks its N-terminus, prevents SCS. This can be overcome by fusing the D-box from Cdc13p, the fission yeast homologue of cyclin B onto the N-terminus of Cut2p (Funabiki *et al.*, 1996).

1.2.5.4 PEST sequences

The PEST motif, containing stretches of amino acids enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) residues, was proposed as an important determinant for rapid degradation (Rogers *et al.*, 1986). This sequence has been identified in a number of short-lived proteins such as yeast G₁ cyclins Cln2p and Cln3p (Yaglom *et al.*, 1995), the transcription factor Gcn4p, ornithine decarboxylase (ODC) (Murukami *et al.*, 1992) and the p53 tumour suppressor protein (Chowdhary

et al., 1994). All of these proteins are known to be degraded by the ubiquitin-dependent proteasome pathway, although ODC does not require ubiquitination for its proteolytic degradation.

Although the mechanism by which these motifs may confer susceptibility to degradation is not fully understood, they may act as targets for serine / threonine protein kinases. Phosphorylation could act as a conditional signal for degradation by inducing a conformational change, thereby exposing another region of the protein for ubiquitination.

The PEST motif has been shown to function as a transplantable proteolytic signal. Transplantation of the PEST motif of Cln3p from *S.cerevisiae* onto β -galactosidase resulted in the rapid ubiquitination and subsequent degradation of this fusion (Yaglom *et al.*, 1995). By contrast, removal of PEST-containing regions from ODC or from NIMA, a cell cycle regulated protein kinase from *Aspergillus nidulans*, stabilised these proteins (Ghoda *et al.*, 1989; Pu and Osmani, 1995).

1.2.6 Deubiquitination

Following protein degradation by the 26S proteasome, cells have a requirement to recycle the polyubiquitin chains thereby producing monomeric ubiquitin. Failure to perform this essential function results in a depletion of the cytoplasmic ubiquitin pool. This potential hazard cannot be overcome by the expression of polyubiquitin genes, since *UBI4* and its orthologues are only induced under conditions of stress. Deubiquitination of ubiquitin protein conjugates is performed by the action of ubiquitin-specific proteases (UBP). In *S.cerevisiae*, there are 17 UBPs, classified by the presence of the 'Cys' and 'His'- boxes (Hochstrasser, 1996) (see Table 1.2).

The *UBP* genes have only been characterised in detail in budding yeast, in which many other enzymes of the ubiquitin system have already been isolated. *UBP1*, encoding a 90kDa enzyme, was isolated by its ability to cleave natural and engineered ubiquitin fusions (Tobias and Varshavsky, 1991). Two additional genes, *UBP2* and

UBP3, were isolated using an *E.coli* based genetic screen. Whereas Ubp2p is capable of cleaving poly-ubiquitin when co-expressed with it in *E.coli*, Ubp3p is not (Baker *et al.*, 1992). Although the enzymes encoded by *UBP2* and *UBP3* exhibit different properties, there is redundancy between members of this family. A yeast mutant carrying a deletion in the genes *UBP1*, *UBP2*, *UBP3* and *YUH1* was viable (Baker *et al.*, 1992) indicating that other Ubp enzymes were capable of fulfilling the tasks performed by these enzymes. Thus it is possible that the different Ubp enzymes may act on different substrates *in vivo*.

Sequence alignment of the amino acid residues encoded by *UBP1-3*, revealed that the similarity exists in two small regions. One of these, the 'Cys-box' contains a conserved cysteine residue, whereas the other region, the 'His-box' contains two histidine residues. As UBPs are known to be cysteine proteases, these residues probably form part of the active site of the enzyme (Baker *et al.*, 1992).

An additional yeast Ubp has been functionally identified based on the presence of these conserved domains. This enzyme, Doa4 (Ubp4), was isolated as a yeast mutant that stabilised the Mat α 2 repressor protein. Mat α 2 is normally rapidly degraded by the ubiquitin pathway, thus implicating Doa4 (Ubp4) directly in proteolysis (Papa and Hochstrasser, 1993).

UBP homologues have been identified in higher organisms. Two of these, the mouse Unp oncogene (Gupta *et al.*, 1993) and the human tre-2 oncogene, a homologue of Doa4, (Heubner *et al.*, 1988; Nakamura *et al.*, 1992) were thought to have regulatory roles. However, both have also been shown to have Ubp activity. The *Drosophila melanogaster* fat facets gene encodes a UBP which is required for cell fate determination in the developing eye (Huang *et al.*, 1995).

By analogy to the ubiquitin conjugating enzymes, the large number of UBPs may reflect a variance in the substrate specificity and function of this family of enzymes. For example, isopeptidase-T, a human UBP homologous to the yeast Ubp14p, is

Table 1.2 The *S.cerevisiae* deubiquitinating enzymes (taken from Hochstrasser 1996).

<u>Protein</u>	<u>kDa</u>	<u>Function</u>
Ubp1	93	Cleaves ubiquitin-protein fusions, not essential
Ubp2	145	Cleaves ubiquitin-protein fusions, not essential
Ubp3	102	Cleaves ubiquitin-protein fusions, not essential
Ubp4/ Doa4	105	Cleaves ubiquitin-protein fusions, required for resistance to stress, degradation of ubiquitin system substrates and maintenance of ubiquitin pools
Ubp5	92	Cleaves ubiquitin-protein fusions, not essential
Ubp6	57	Not determined
Ubp7	123	Cleaves ubiquitin-protein fusions
Ubp8	54	Not determined, mutant has slight growth defect
Ubp9	86	Cleaves ubiquitin-protein fusions
Ubp10/ Str4	90	Mutant is defective in chromatin-mediated silencing
Ubp11	83	Not determined
Ubp12	143	Not determined
Ubp13	84	Not determined
Ubp14	91	Cleaves ubiquitin oligomers with free C-terminus <i>in vivo</i> and <i>in vitro</i> . Functional homologue of mammalian isopeptidase-T. Required for degradation of MAT α 2.
Ubp15	143	Mutant has slight growth defect
Ubp16	57	Not determined
Yuh1	26	Cleaves ubiquitin from small peptide adducts

unable to degrade poly-ubiquitin which doesn't have a free C-terminus. This function may be performed by a Doa4 homologue, since a deletion of Doa4 results in the accumulation of small ubiquitinated peptides. By contrast, polyubiquitin with a free C-terminus is efficiently degraded by isopeptidase-T (Wilkinson *et al.*, 1995).

An increase in the rate of processing of poly-ubiquitinated proteins, in the presence of the 26 S proteasome, suggested that there may be a deubiquitination activity associated with this complex (Eytan *et al.*, 1993; Papa and Hochstrasser, 1993). This has recently been confirmed since isopeptidase activity has been found to be associated with the bovine 26S complex (Lam *et al.*, 1997). The molecular weight (37kDa) of this proteasomal enzyme is much less than that of Doa4 (100kDa) and Isopeptidase-T (93kDa) suggesting that the activity is not due to a homologue of either of these enzymes. Also, the action of the enzyme removes ubiquitin from the distal end of Lys⁴⁸ linked polyubiquitin chains, an activity distinct from that of other UBPs. An isopeptidase of this type has also been isolated from erythrocytes which, in addition to possessing the above isopeptidase activity, possesses C-terminal hydrolase activity which liberates Ub when it is attached to the N-terminus of proteins (Moskovitz *et al.*, 1994).

Ubp enzymes have recently been implicated in the control of gene expression by silencing in heterochromatin. The Ubp3 enzyme has been shown to interact directly with Sir4, one of a set of proteins required for the silencing of transcription at the mating type loci and telomeres in *S.cerevisiae* (Mozaed and Johnson, 1996). Deletion of the *UBP3* gene results in markedly improved silencing at both the mating loci and at telomeres. Enhancement of silencing has also been shown to occur in *Drosophila* by inactivation of the D-Ubp-64E gene, which, while not being an orthologue of Ubp3, has a similar phenotype to a mutation in Ubp3 (Henchoz *et al.*, 1996).

1.2.6.1 Regulation of proteolysis by deubiquitination

As discussed above, different Ubp enzymes act to recycle ubiquitin in different ways. The 26S-associated isopeptidase activity removes ubiquitin moieties from the distal

end of a poly-ubiquitin chain (Lam *et al.*, 1997), whereas isopeptidase-T acts only on the free C-terminal glycine residue produced following cleavage of the bond between the ubiquitin and the protein substrate (Wilkinson *et al.*, 1995).

The presence of an activity which can disassemble poly-ubiquitin chains from their N-terminus provides a potential means of regulating protein turnover. This regulation would be advantageous in that degradation of proteins which were ubiquitinated with a small number of ubiquitin moieties, would be less likely to occur than those proteins which were poly-ubiquitinated. In addition, this activity would not require any C-terminal hydrolase activity for removal of the protein substrate and exposure of a free Gly⁷⁶ residue.

1.2.7 Intracellular functions associated with ubiquitination

Ubiquitination seems to be mainly associated with the degradation of proteins by the 26S proteasome. However, there are several other functions for ubiquitination including an involvement in other pathways of protein degradation.

1.2.7.1 Endocytosis

The use of *S.cerevisiae* as a model system for the study of the endosomal pathway has demonstrated that in response to ligand binding, ubiquitination of the cognate receptor molecule acts as a signal for the internalisation and subsequent degradation of at least one cell surface pheromone receptor, Ste2p. (Hicke and Riezman, 1996). Degradation of this protein requires the presence of active vacuolar proteases within the cell (Schandel and Jenness, 1994; Klionsky *et al.*, 1990). In mutants lacking these active proteases, ligand induced internalisation of Ste2p resulted in an accumulation of ubiquitinated receptor (Hicke and Riezman, 1996).

In contrast to the generalised view that ubiquitination serves to target proteins for degradation via the 26S proteasome, the above result demonstrates that this is not always the case. In normal mammalian cells for example, ubiquitin-protein conju-

gates are found in the primary lysosome granules (Laszlo *et al.*, 1991). It therefore seems likely that in addition to the other targeting signals, ubiquitination can act as a signal for entry into this degradation pathway. Indeed, ubiquitin has been found to be associated with lysosomal-like organelles in certain neurodegenerative disorders (Laszlo *et al.*, 1991).

The use of specific inhibitors of lysosomal / vacuolar proteases has demonstrated that in different organisms, the degradation of long-lived proteins is facilitated by different pathways. In cultured mammalian cells, inhibition of the peptidase activities of the 26S proteasome stabilised both long-lived and short-lived proteins (Rock *et al.*, 1994). In yeast, this treatment only stabilised the turnover of short-lived proteins (Lee and Goldberg, 1996). When yeast cells were treated with PMSF, an inhibitor of vacuolar proteases, however, the degradation of long-lived proteins but not short-lived proteins was blocked. Therefore, separate pathways are used for the degradation of long-lived proteins in yeast and mammalian cells

1.2.7.2 The UFD pathway for protein degradation

In addition to the N-end rule, the UFD (ubiquitin fusion degradation) pathway in *S.cerevisiae* also plays a role in proteolysis (Johnson *et al.*, 1995). Though no natural substrates of this pathway have yet been discovered, a screen to look for the stabilisation of artificial substrates has defined 5 genes whose products are involved. Of these 5 *UFD* genes only one, *UFD1*, is essential. Two, *UFD2* and *UFD4*, are involved in promoting the polyubiquitination of different lysine residues internal to the ubiquitin molecule at positions 29 and 48 respectively. A mutation in *UFD5* stabilises substrates of both N-end rule and *UFD* pathways indicating that these two pathways have elements in common.

Like the 'N'-end rule pathway, the Ufd pathway seems to play a minor role in protein turnover within the cell (Johnson *et al.*, 1995). Yet the N-end rule pathway has been found to function in bacteria, yeast and mammalian cells. It is therefore possible that the *UFD* pathway exists in these organisms also.

1.3 The 26S Proteasome

Following polyubiquitination, substrates appear to be rapidly degraded in an ATP-dependent reaction involving a large multiprotein complex. This complex was first isolated from rabbit reticulocytes and was discovered by its ability to degrade labelled Ub-lysozyme conjugates (Hough *et al.*, 1986). Due to its sedimentation coefficient, this large 1500kDa complex has been termed the 26S proteasome and has so far been identified only in eukaryotic cells.

This large 26S complex comprises two distinct smaller complexes, a 20S catalytic core and a 19S regulatory complex, which are required to be associated in order to degrade Ub-conjugated substrates (Coux *et al.*, 1996; Hochstrasser, 1996; Hoffman and Rechsteiner, 1996). The latter complex is one of a number of complexes which have been found to alter the activity of the 20S proteasome. These complexes will be subsequently discussed. An electron density map, and an interpretation of this image (after Rubin *et al.*, 1995), is shown in Figure 1.2

The relationship between the 20S proteasome and the 26S complex became clear when it was demonstrated that the 20S proteasome was incorporated into a larger complex as one of 3 conjugate degrading factors (CF-1, 2 and 3) that were required for the assembly of the 26S complex in an ATP-dependent reaction (Ganoth *et al.*, 1988; Eytan *et al.*, 1989; Driscoll and Goldberg, 1990). The 20S proteasome was equivalent to the factor CF-3), whereas CF-1 and CF-2 were an activator (the 19S complex) and an inhibitor of the peptidase activity of the CF-3, respectively.

CF-1 is identical to the μ -particle from *Drosophila* (Udvardy, 1993), a complex which has no ATPase activity *per se*, but which is incorporated into the 26S proteasome in an ATP-dependent reaction. By contrast, CF-2 has been identified as a complex containing multiple copies of the enzyme δ -aminolevulinic acid dehydratase, an enzyme required for heme biosynthesis (Driscoll *et al.*, 1992).

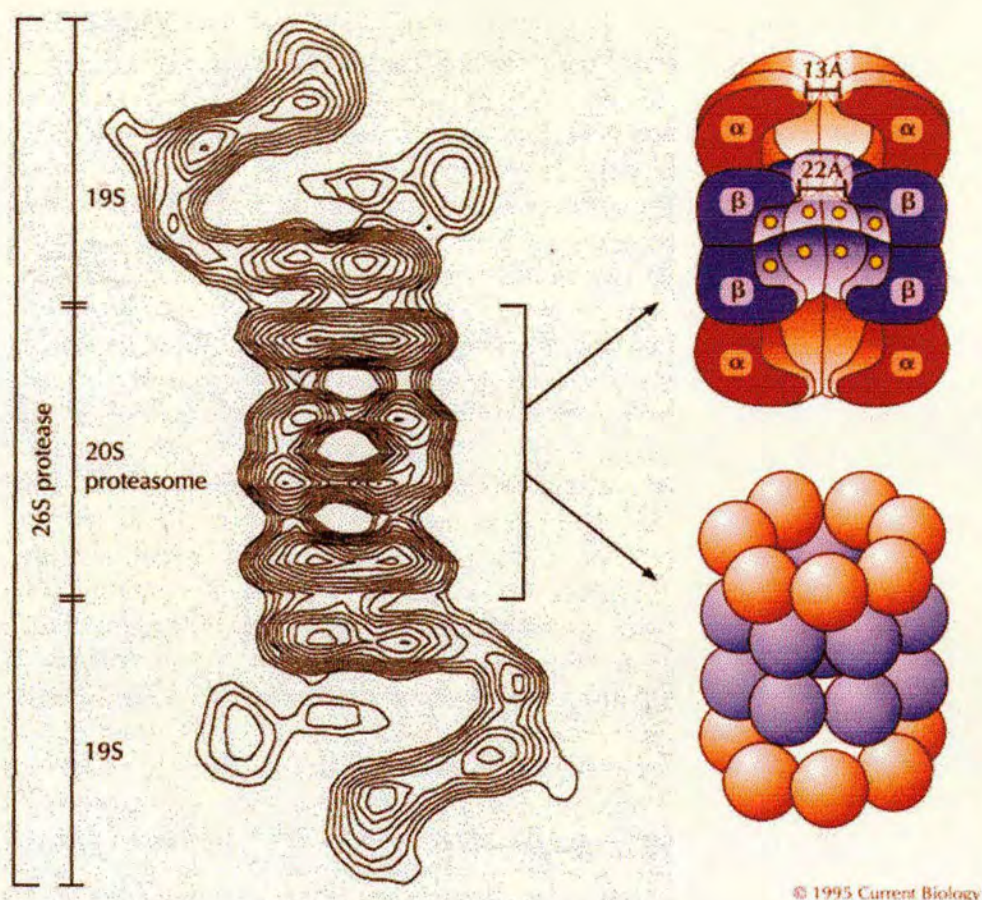


Figure 1.2 Structural features of the 26S proteasome (taken from Rubin and Finley, 1995).

Left, contour plot of the 26S proteasome derived from electron microscopy and image analysis. Top right, schematic cross-section of the proteasome, showing the location of α subunits (red) and β subunits (blue) and peptidase active sites (yellow). Bottom right, schematic view of subunit arrangement in the 20S proteasome.

1.3.1 The 20S proteasome

The 20S proteasome or multicatalytic proteinase complex (MCP), was first described in bovine pituitaries as a high molecular weight protease (700kDa) comprising low molecular weight (20-34kDa) non-identical subunits (Orlowski and Wilk, 1981). This abundant complex, found in all eukaryotes as well as in the archaebacterium *Thermoplasma acidophilum* (Dahlmann *et al.*, 1989) and in the eubacterium *Rhodococcus* (Tamura *et al.*, 1995), comprises 0.5-1% of total protein in tissue homogenates (Orlowski, 1990).

Although having been isolated in many tissues, and having been assigned a variety of names including 'proteinase yscE' and 'macropain', the appearance of the MCP, from EM images, was that of a cylindrical structure of 16nm in length, which surrounded a pore with a diameter of 4nm. This structure was very similar to that of the 'prosome', a complex thought to be associated with the regulation of mRNA transcription (Arrigo *et al.*, 1988). Both complexes have a similar size, shape, subunit composition and proteolytic activity. In addition, there is a similar immunological cross-reactivity between particles isolated from different species (Orlowski, 1990).

1.3.1.1 Structure, biogenesis and enzymatic properties of the 20S proteasome

The structure of the eukaryotic MCP was first elucidated by electron microscopy (EM) (Baumeister *et al.*, 1988). These and subsequent analyses revealed that it is arranged as a hollow cylinder comprising 4 rings, each of which contains 7 subunits (see Figure 1.2). The inner β rings of this structure were found to be rotated approximately 25° with respect to the outer α rings (Baumeister *et al.*, 1988). EM study of the proteasome from *T.acidophilum* confirmed that the subunit arrangement was very similar to that of the eukaryotic proteasome (Puhler *et al.*, 1992). The positions of each of the 28 subunits in the complex has been determined by chemical crosslinking of neighbouring subunits (Kopp *et al.*, 1997).

The crystal structure of the yeast 20S proteasome has also been solved (Groll *et al.*, 1997). This structure has highlighted that in contrast to the previously observed pore at either end of the 20S complex in the archaebacterial proteasome, no pore is present in the *S.cerevisiae* particle. Side chains from the α subunits at either end of the 20S complex interdigitate to prevent access to the catalytic chamber formed by the β -subunits (Groll *et al.*, 1997).

Using 2-Dimensional PAGE, all of the subunits of the MCP have been shown to have a molecular weight of 20-35kDa. Sequence analysis of the subunits of the proteasome from human, rat, budding yeast, and *Drosophila*, indicate that they are 20-40% identical to each other. These deduced sequences have been classified as either α or β according to their similarity to the subunits from *T.acidophilum* (Puhler *et al.*, 1992). Subunits of the β -type form the two inner rings, and contain the catalytic activity, whereas the outer rings are formed by the α -subunits.

The first step in assembly of the eukaryotic 20S proteasome is the formation of ring structures by the α subunits, which by doing so, provide docking sites for the different β subunits (Schmidt *et al.*, 1997). Docking of the β subunits leads to formation of a double ring structure representing a half proteasome. Two of these half proteasomes associate to form a proteolytically inactive 20S precursor. Catalytic β subunit precursors are then processed leading to the mature, active 20S proteasome.

The 20S proteasome is an N-terminal nucleophile hydrolase. This nucleophile is a threonine residue which is exposed following autocatalytic cleavage of a Gly-Thr bond in the propeptide precursors of the mature β subunits (Seemüller *et al.*, 1996). All of the catalytic β subunits are synthesised as inactive precursors, and require the α subunits for processing and assembly (Chen and Hochstrasser, 1996; Seemüller *et al.*, 1996). Processing of 3 of the β subunits β 1/Pre3, β 2/Pup1 and β 5/Pre2, exposes a threonine residue, which acts as an N-terminal nucleophile, the active site of the catalytic subunits (Seemüller *et al.*, 1996).

There are 5 proteolytic activities associated with the eukaryotic MCP *in vitro* which include trypsin-like (T-L), chymotrypsin-like (CT-L) peptidyl-glutamyl hydrolysing

activity (PGPH) and branched-chain amino acid preferring activity (BRAAP) (Rivett, 1993). By contrast, the *Thermophilus* proteasome only has CT-L activity (Seemüller *et al.*, 1995). The activities have been shown to be catalysed by different subunits within the complex since they respond differently to various activators and inhibitors. The β -lactone derivative, lactacystin, inhibits all of the proteasomal enzymatic activities, and as such has been used to determine the involvement of the proteasome in all forms of intracellular proteolysis (Fenteany *et al.*, 1995).

Deletion of subunits of the 20S proteasome in *S.cerevisiae* suggested that all but one of the subunits, Y13, an α -type subunit, were essential (Emori *et al.*, 1991; Heinemeyer *et al.*, 1994). However recent work in this laboratory has shown that a *ts* mutation in the *S.pombe* homologue of this subunit is lethal at the restrictive temperature (O. Rooyackers, unpublished results). This latter result suggests that all of the subunits of the 20S proteasome are essential for full function of the complex.

Although there are now known to be 14 distinct subunits which are incorporated into the 20S proteasome in yeast (Heinemeyer *et al.*, 1994), the situation in mammalian cells is more complex. Upon stimulation with the regulatory cytokine interferon- γ (IFN- γ), 3 proteins LMP2, LMP7, and MECL1 are incorporated into this complex in place of their human proteasomal homologues, subunits X(δ) and Y(MB1) and Z respectively (Goldberg and Rock, 1992, Akiyama *et al.*, 1994). These subunits function to alter the proteolytic activity and composition of the proteasome, and the nature of the peptides produced by proteolytic cleavage (Eggers *et al.*, 1995; Groettrup *et al.*, 1995). Unlike their normal counterparts, the IFN- γ induced subunits are not essential (Früh *et al.*, 1994)

1.3.1.2 Localisation of the 20S proteasome

Studies using EM and immunocytochemistry have localised the proteasome to both the nucleus and the cytoplasm in eukaryotes (Akhayat *et al.*, 1987; Arrigo *et al.*, 1988). It has been found associated with the ER, and cytoskeleton in *Drosophila melanogaster* (Kloetzel *et al.*, 1987) and with the intermediate filament (IF) network

in avian erythroblasts (Martins de Sa *et al.*, 1988; Olink-Coux *et al.*, 1992). Using EM, the proteasome from rat liver was observed to be associated with the nucleus, and diffusely distributed in the cytoplasm, where it was associated with the ER (Rivett *et al.*, 1992).

A number of groups have observed a change in distribution of the proteasome during different stages of the cell cycle or during development. Cytoplasmic proteasomes in immortalised ovarian granulosa cells during interphase, were found to be localised in the perichromosomal area during prophase (Amsterdam *et al.*, 1993). In metaphase, these proteasomes were concentrated in the vicinity of spindle microtubules. This phenomenon also occurred during oogenesis in the newt, suggesting that rapid transfer is an important facet in proper proteasome function (Gautier *et al.*, 1988). The presence of a nuclear localisation signal (NLS) on at least 4 subunits, 2 α -type and 2 β -type (Rivett and Knecht, 1993; Heinemeyer *et al.*, 1994), may be important in the re-localisation of proteasomes during the cell cycle.

1.3.1.3 *In vivo* functions of the 20S proteasome

1.3.1.3.1 MCP and antigen presentation

The immune response in mammalian cells relies on the identification of foreign antigens by cytotoxic T-cells, and their subsequent phagocytosis. The recognition of these antigen-presenting cells (APC) is now known to be facilitated by the production of antigenic peptides by the eukaryotic 20S proteasome in association with the PA28/11S regulator (see section 1.3.2) (Mott *et al.*, 1994; Hoffman and Rechsteiner, 1994). Antigenic peptides are transported into the ER by the MHC class II encoded TAP transporter proteins which are members of the ABC family of transmembrane transporters (Deverson *et al.*, 1988, Trowsdale *et al.*, 1989). Here they are complexed with a major histocompatibility complex (MHC) class I molecule. From the ER, they are translocated out of the ER, through the secretory pathway, to the cell surface, where they are presented on the surface of an APC.

Induction of proteasomal subunit expression in fibroblasts by interferon- γ , resulted in the replacement of three subunits by two MHC-encoded proteins LMP2 and LMP7 and a third non-MHC-encoded subunit MECL1 (Goldberg and Rock, 1992; Yang *et al.*, 1992). The newly induced subunits have a high (76%) homology to the replaced sub-units X (δ), Y (MB1) and Z. This induction affects the proteolytic function of the proteasome by enhancing the T-L, CT-L and BRAAP activity and decreasing the (PGPH) activity. The increase in this latter activity generates peptides which are more easily bound by MHC class I molecules in the ER, thereby increasing the efficiency of the immune response.

1.3.2 Regulators of the 20S proteasome

Although the 20S proteasome possesses all of the known endo-peptidase activities associated with the 26S complex, its activity must be regulated. At least 3 regulators of the 20S proteasome are known. Two of these, the 11S regulator (PA28) and the 19S regulatory complex (PA700) are dependent only on the 20S proteasome for binding. Of these, only the 19S regulator requires ATP for its association with the 20S proteasome. The other regulator, 'the modulator', is dependent on the binding of the 19S regulatory complex. As such, it will be discussed as a regulator of the 26S complex.

1.3.2.1 The PA28/ 11S regulator

As mentioned in section 1.3.1.3.1, antigen presentation by the 20S proteasome is regulated by the association of this complex with the 11S regulator complex, also known as PA28. This 180kDa complex was isolated from bovine red blood cells (Chu-Ping *et al.*, 1992) and later from human red blood cells (Dubiel *et al.*, 1992). The hexameric complex is made up of two closely related subunits PA28 α and PA28 β , which form a ring structure and bind to the α ring of the proteasome (Gray *et al.*, 1994). Like LMP2, LMP7 and MECL1, PA28 α and PA28 β are induced by IFN-

γ . The binding of the complex, which occurs in the absence of ATP, changes the quality and quantity of peptides produced (Groettrup *et al.*, 1994).

A third member of the PA28 family, the Ki antigen (PA28 γ), has also been found to co-immunoprecipitate and to associate reversibly with the 20S proteasome (Tanahashi *et al.*, 1997). However, whereas treatment of cells with IFN- γ markedly induces the expression of PA28 α and PA28 β , the level of PA28 γ protein drops dramatically upon IFN- γ treatment (Ahn *et al.*, 1995). The function of this protein is as yet unknown, but the reciprocal expression of PA28 family members upon IFN- γ treatment suggests a distinct function for these proteins.

Analysis of the PA28 α subunit has revealed the presence of a C-terminal KEKE motif rich in lysine and glutamic acid residues (Groettrup *et al.*, 1996). This motif is also present in two neighbouring α subunits of the 20S proteasome (Y13 / C9, and XAPC7) (Realini *et al.*, 1994), and in S12 of the 19S regulatory complex (Dubiel *et al.*, 1995). The significance of this motif is unknown, although the its presence on two neighbouring α subunits suggests that it may form a local centre for association of the 19S and PA28 regulatory complexes (Realini *et al.*, 1994). Indeed, treatment of the PA28 complex with carboxypeptidase, which removes the C-terminal KEKE motif from PA28 α , abolishes the interaction of PA28 with the 20S complex.

Another proteasome α subunit, C2, is important in the binding of PA28 to the proteasome (Kania *et al.*, 1996). Pre-incubation of the proteasome with an antibody against the C2 subunit prevented association of PA28 with the proteasome, but not with the 19S complex, indicating that these regulatory complexes utilised different binding sites on the 20S proteasome (Kania *et al.*, 1996). It is not known whether a PA28 complex binds to each end of the 20S proteasome at the same time, or whether it only binds to one end, allowing the other end to be occupied by another activator such as the 19S regulator (see below). However, maximal proteolytic stimulation occurs at a PA28:20S proteasome ratio of 1:1 (Kuehn and Dahlmann, 1996).

Recent work has implicated the human immunodeficiency virus (HIV) Tat protein as an inhibitor of the interaction between the PA28 / 11S regulator and the proteasome (Seeger *et al.*, 1997). Proteolytic activity of the proteasome was inhibited by

incubation of the proteasome with Tat. The results suggested that Tat competed with PA28 for binding sites on the proteasome, thereby diminishing the potential immune response by HIV-infected cells.

1.3.3 PA700, the 19S regulatory complex

Although the 20S proteasome is essential for the degradation of ubiquitinated proteins, it has no dependency on ATP for this degradation reaction. ATP is required for the association of the 19S regulatory complex with the 20S particle to produce the 26S complex (Hough *et al.*, 1987). When purified, this complex degrades polyubiquitin-protein conjugates in an ATP-dependent manner.

The identity of a number of subunits of the 19S cap complex has been obtained by peptide sequencing of purified subunits followed by cDNA cloning of the corresponding genes (Dubiel *et al.*, 1992; De Martino *et al.*, 1994; Dubiel *et al.*, 1995). This hetero-oligomeric complex has been found to comprise more than 16 subunits with a molecular weight between 35-110kDa, originally designated S1-S15. Individual subunits of the cap complex are either ATPases, belonging to a multigene family present in prokaryotes and eukaryotes, or non-ATPases. The identity of all known subunits of the 19S regulatory complex is shown in Table 1.3.

1.3.3.1 Non-ATPase subunits

Many of the non-ATPase subunits of the 19S regulatory complex have now been identified (Dubiel *et al.*, 1995; Richmond *et al.*, 1997) (and see Table 1.3). Analysis of the genes encoding these subunits from both yeast and mammalian cells suggest that while the subunits are not as conserved in sequence as are the ATPases, (see Section 1.3.3.2) their functional conservation is strong (Dubiel *et al.*, 1995). Despite this, the function of many of these subunits remains unknown.

The largest subunit (S1) of the 26S proteasome, is encoded by the yeast *SEN3* gene (De Marini *et al.*, 1995). The product of this essential gene has a molecular weight of 112kDa. Although the only function ascribed to this subunit is its involvement with

tRNA splicing, a mutation in *SEN3* shows synthetic lethality with a mutation in the *NIN1* gene, which encodes S14 of the regulatory complex (Kominami *et al.*, 1995; Gordon *et al.*, 1996), thus indicating a functional interaction between these two subunits. A functional homologue of *SEN3* has also been isolated from human cells (Yokota *et al.*, 1996).

S2 was isolated by its interaction with the tumour necrosis factor (TNF) receptor, and given the name TRAP2 (Tsurumi *et al.*, 1996). Since then, homologues have been found in *S.pombe* (Mts4p) and *S.cerevisiae* (Nas1p) (Tsurumi *et al.*, 1996; Wilkinson *et al.*, 1997; Hampton *et al.*, 1996). Both of these yeast proteins are 45% identical at the amino acid level to the human homologue. Although the peptide sequences of these subunits have diverged significantly, human p97 can suppress the phenotype of budding yeast *nas1* deletion mutants, indicating a functional conservation of these subunits (Tsurumi *et al.*, 1996).

The interaction between S2 and the TNF receptor suggested an association between the 26S proteasome and intracellular membranes. This was verified by the discovery that this subunit is involved in the regulation of the mevalonate pathway in *S.cerevisiae* by ER associated degradation of the key enzyme 3-hydroxy 3-methyl glutaryl CoA reductase (HMG CoA reductase), an integral ER membrane protein (Hampton *et al.*, 1996).

The gene encoding the *S.pombe* homologue of S2, Mts4p, was isolated in the same screen as that for Mts2p. These proteins have been shown to interact by genetic, physical and *in vitro* analyses. The *mts2-1* and *mts4-1* mutants show synthetic lethality. This genetic interaction has been confirmed by the ability of an overexpression of *mts2*⁺ to partially suppress the *ts* defect of the *mts4-1* mutant (Wilkinson *et al.*, 1997). Furthermore, physical interactions between these two proteins has been demonstrated by two-hybrid analysis and by *in vitro* binding studies. This is the first evidence of an *in vivo* interaction between ATPase and non-ATPase subunits of the 26S proteasome.

Table 1.3 Subunits of the 19S regulatory complex

<u>Human</u>	<u>kDa</u>	<u><i>S.cerevisiae</i></u>	<u><i>S.pombe</i>/Other</u>	<u>References</u>
S1	112	Sen3		de Marini <i>et al.</i> , 1995 Yokota <i>et al.</i> , 1996
S2	97	Nas1/ Hrd2	Mts4	Tsurumi <i>et al.</i> , 1996 Hampton <i>et al.</i> , 1996 Wilkinson <i>et al.</i> , 1997
S3	58	Sun2		de Martino <i>et al.</i> , 1994 Kawamura <i>et al.</i> , 1996 Kominami <i>et al.</i> , 1997
*S4	56	Yta5	Mts2	Dubiel <i>et al.</i> , 1992 Gordon <i>et al.</i> , 1993 Schnall <i>et al.</i> , 1994
S5a	50	Sun1	Mcb1/Mbp1	Deveraux <i>et al.</i> , 1994 van Nocker <i>et al.</i> , 1996 Kominami <i>et al.</i> , 1997
S5b	50			Deveraux <i>et al.</i> , 1995
*S6/ TBP7	47	Tbp7/ Yta2		Ohana <i>et al.</i> , 1993 Dubiel <i>et al.</i> , 1994 Schnall <i>et al.</i> , 1994
*S6'	46	Tbp1		Nelbock <i>et al.</i> , 1990 Ohana <i>et al.</i> , 1993
*S7/ MSS1	47	Cim5	Aps1	Irie <i>et al.</i> , 1991 Shibuya <i>et al.</i> , 1992 Dubiel <i>et al.</i> , 1993 Ghislain <i>et al.</i> , 1993
*S8/ p45/ Trip1	45	Cim3/ Sug1	Let1	Swaffield <i>et al.</i> , 1992 Ghislain <i>et al.</i> , 1993 Michael <i>et al.</i> , 1994 Akiyama <i>et al.</i> , 1995 Lee <i>et al.</i> , 1995
Centractin				Richmond <i>et al.</i> , 1997
S9	46			Hoffman <i>et al.</i> , 1997
S10a				Richmond <i>et al.</i> , 1997
*S10b	44-49	Sug2		Bauer <i>et al.</i> , 1996 Fujiwara <i>et al.</i> , 1996 Russell <i>et al.</i> , 1996
S11	40			Dubiel <i>et al.</i> , 1993

S12	35-37		Pad1/ Mov34	Dubiel <i>et al.</i> , 1995 Shimanuki <i>et al.</i> , 1995
S14		Nin1	Mts3	Nisogi <i>et al.</i> , 1992 Kominami <i>et al.</i> , 1995 Gordon <i>et al.</i> , 1996
S?			Mts1	Dubiel <i>et al.</i> , 1995 C. Gordon, unpublished

Other proteasomal proteins

p37	Isopeptidase		Lam <i>et al.</i> , 1997
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*= 19S subunit with ATPase domain

There is a low level of similarity between S1 and S2 subunits, in a region of about 400 amino acid residues which contains nine repeats of 35-40 residues (Lupas and Baumeister, 1997). The conserved part of this repeat is centred around an alternating pattern of large aliphatic residues. The alternating β -sheets and α -helices formed by these repeats are thought to form a structure which may act to recognise damaged or partially unfolded proteins. This configuration is also seen in BimE, a component of the anaphase promoting complex (APC), which ubiquitinates mitotic cyclin B and targets it for degradation by the 26S proteasome (King *et al.*, 1996).

Binding of ubiquitin-protein conjugates was thought to be carried out specifically by S5a (Deveraux *et al.*, 1994). Surprisingly, however, disruption of the *S.cerevisiae* homologue, *MCBI*, was not lethal, indicating that the function of this subunit was not essential and that another 19S subunit may be involved in the recognition and binding of polyubiquitinated substrates (van Nocker *et al.*, 1996a). As yet, no other ubiquitin-binding subunits have been found, though the recent finding of ubiquitin isopeptidase activity associated with the 26S proteasome suggests that another subunit may carry out the ubiquitin-binding function (Lam *et al.*, 1997). It may be that S5a is only capable of binding certain forms of ubiquitin conjugates such as those produced via Lys¹¹, Lys⁴⁸ or Lys⁶³ linkages (Beal *et al.*, 1996; van Nocker *et al.*, 1996b).

The peptide sequence of the product of the *S.pombe mts3⁺* gene is very similar to that generated from digestion of the human S14 (W. Dubiel, pers. comm.). The *mts3-1* mutant was isolated in the same screen as *mts2-1*, which carries a mutation in S4 of the 26S proteasome (Gordon *et al.*, 1993; Gordon *et al.*, 1996). The phenotype of a conditional mutation in *mts3⁺* is similar to that of the *mts2-1* mutant at the restrictive temperature. However, unlike the *mts2-1* at the restrictive temperature, the *mts3-1* mutant undergoes endoreplication, suggesting that the product of *mts3⁺* is involved in the control of DNA replication (Gordon *et al.*, 1996).

A functional homologue of Mts3p exists in *S.cerevisiae*. This protein, the product of the *NIN1* gene (Nisogi *et al.*, 1992) bears a limited (36%) homology to Mts3p. However, overexpression of *NIN1* can rescue the *ts* defect of the *mts3-1* mutant, as well as

a deletion of the *mts3⁺* ORF, thereby demonstrating that *NINI* is the functional homologue of the *S.pombe mts3⁺* gene (Gordon *et al.*, 1996)

Subunits S5b (Deveraux *et al.*, 1995), S9 (Hoffman *et al.*, 1997), S11 (Dubiel *et al.*, 1993) and S14 (Dubiel *et al.*, 1993), have no function assigned to them as yet. S5b, isolated from human erythrocytes, was found to be enriched in di-leucine repeats, though the significance of this motif is unknown. No homologue of this subunit has been found in either *S.pombe* or *S.cerevisiae*, suggesting that S5b may not be in the 26S proteasome (W. Dubiel, pers. comm.). Another subunit has been identified as centractin, a component of the dynactin complex (Richmond *et al.*, 1997). The presence of this subunit in reticulocyte 26S proteasome extracts may represent a means for the proteasome to interact with the cytoskeletal network.

1.3.3.2 ATPase subunits

Six ATPases have been identified as subunits of the 26S proteasome (see Table 1.3). They are all members of the S4 family of ATPases, which is a subset of a much larger family of ATPases, the AAA family (Confalonieri and Duguet, 1995). Members of the AAA family are characterised by the presence of one or two copies of a Walker 'A' box, containing the phosphate-binding 'P-loop' motif (Saraste, 1990) and the Walker 'B' box (Walker *et al.*, 1982). All members of the proteasomal ATPases have one copy of this motif. Sequencing of the gene encoding the human S4 subunit, followed by database analysis of its peptide sequence, revealed a strong homology between this peptide and those encoded by TBP-1, TBP-7, MSS1 and *SUG1* (Dubiel *et al.*, 1992). All of these proteins, as well as Sug2p (Fujiwara *et al.*, 1996; Russell *et al.*, 1996), are now known to be present as subunits in the 26S proteasome.

The first member of this family, TBP-1, was isolated on the basis of its interaction with the human immunodeficiency virus (HIV) Tat protein (Nelbock *et al.*, 1990). TBP-7 was subsequently isolated in the same way (Ohana *et al.*, 1993). Like TBP-1 and TBP-7, the next two members, MSS1 and Sug1p were thought to function as

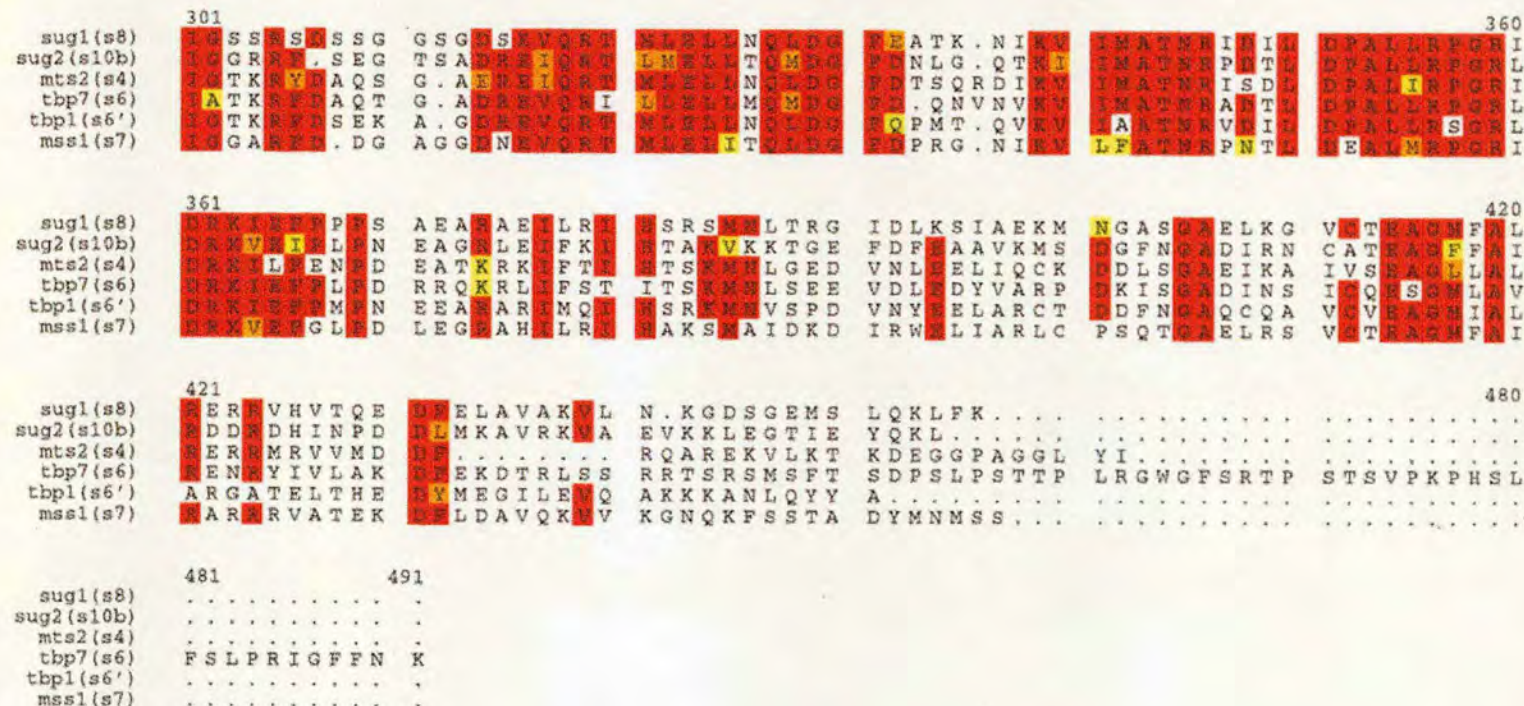


Figure 1.3 Alignment of peptide sequences of yeast ATPases subunits of the 26S proteasome.

Peptide sequences were fetched from the SWISSPROT database and aligned using the LINEUP and PILEUP and PRETTYBOX programs in the UWGCG suite. The plurality used was 3.1. Amino acid residues highlighted in red are identical. Those in orange are very similar in shape and charge, and those in yellow belong to the same family. The Sug1, Mts2 and MSS1 sequences are from *S.pombe*, whereas Tbp1, Tbp7 and Sug2 are from *S.cerevisiae*. Accession numbers: Sug1 P46836, Sug2 P53549, Mts2 P36612, Tbp7 P33298, Tbp1 P33297.

transcriptional regulators. MSS1, was found to be a positive modulator of HIV Tat-mediated transactivation, and suppressed a mutation in a Cdc2 / Cdc28-like protein-kinase (Shibuya *et al.*, 1992). Sug1p, however, was isolated as a suppressor of a C-terminal deletion in the *S.cerevisiae* Gal4 transcription factor (Swaffield *et al.*, 1992). More recently, a second suppressor of the Gal4p deletion, *SUG2*, was found. The product of this gene has been found to strongly resemble the archetypal S4 subunit of the 26S proteasome (Russell *et al.*, 1996). All 6 of these ATPases are now known to be subunits of the 26S proteasome (Dubiel *et al.*, 1992, 1993, 1994, 1995; Rubin *et al.*, 1996; Richmond *et al.*, 1997).

The homology of these ATPases to each other is extensive. Each family member possesses a non-conserved N-terminal domain upstream of a very highly conserved 230 amino acid domain. This conserved region has a functional nucleotide binding site comprising the Walker A and Walker B boxes (Walker *et al.*, 1982). In addition, two other regions, known to be common to a family of RNA / DNA helicases, are also present (Gorbalenya *et al.*, 1989). The family of proteasomal ATPases is shown in Figure 1.3.

The N-terminus of all of the putative proteasomal ATPases possesses a potential amphipathic helix which is thought to be responsible for the formation of coiled-coil regions. In the case of TBP-1 and TBP-7, which form a heterodimer, these regions are required for this interaction (Ohana *et al.*, 1993). The leucine zipper motif found in the N-termini of TBP-1 and TBP-7 is also found in MSS1 and Sug1p. All four of these proteins were thought to be involved in transcriptional regulation, although more recent evidence suggest a primary role for all of these proteins as part of the 19S regulatory complex (Rubin *et al.*, 1996; Richmond *et al.*, 1997). The leucine-zipper may act to facilitate the capture of potential substrates for degradation.

The use of the yeasts *S.cerevisiae* and *S.pombe* has allowed genetic analysis of the genes encoding these ATPase subunits. A conditional mutation in *mts2*⁺, which encodes the fission yeast homologue of the human S4 subunit, caused cells carrying this mutation to arrest at the metaphase stage of mitosis when incubated at the restrictive temperature (Gordon *et al.*, 1993). A similar phenotype was observed in *S.cerevisiae*

cells carrying *ts* mutations in two genes encoding the ATPase subunits Cim3p (Sug1p) and Cim5p (Mss1p) (Ghislain *et al.*, 1993).

The phenotype of a conditional mutation in Sug2p, the sixth ATPase subunit of the 26S proteasome, is different to those described above. Cells carrying this mutation arrested at the G₂ stage of the cell cycle, with replicated but unsegregated DNA (McDonald and Byers, 1997). Only one spindle pole body (SPB) was visible, suggesting that Sug2p acted at a stage earlier than Sug1p, Mss1p and Mts2p. The phenotypes of conditional mutations in other proteasomal ATPase encoding genes, such as *mts2*⁺, are identical to those of a disruption of the same gene, and result in a loss of function. As yet, however, the phenotype of a disruption of the gene encoding Sug2p has not been studied, but it might be predicted that this phenotype would resemble that of a disruption of the *mts2*⁺ gene, in that a loss of function would result from this disruption

1.3.3.3 Function of the 19S regulatory complex

The assembly of the 19S complex with the 20S requires ATP (Hough *et al.*, 1986). The absence of ATP, or the replacement of ATP by non-hydrolysable analogues prevents complex formation and conjugate degradation. It seems likely, therefore, that one of the primary functions of the ATPases within the 19S complex is in the formation and maintenance of the 26S complex from the two sub-complexes. Moreover, each ATPase is essential and cannot be replaced by overexpression of another. Why then are there 6 ATPases?

Although all of the putative ATPase subunits bear a close resemblance to each other, only one of these, the S4 subunit, has been shown to have ATPase activity (Lucero *et al.*, 1995). Given the similarity of all of the ATPases to each other, it is likely that most, if not all, of them, have ATPase activity. The 26S proteasome also possesses GTPase, CTPase and UTPase activity (Hoffman and Rechsteiner, 1996). This suggests that one or all of the remaining ATPases are capable of hydrolysing these nucleotides.

Studies of the archaeobacterial 20S proteasome from *T.acidophilum* indicate that only fully unfolded proteins can enter the narrow (4nm) pore in either end of the 20S complex (Lowe *et al.*, 1995). A further mechanism may therefore be required for the complete unfolding of misfolded proteins. The energy produced by nucleotide hydrolysis may therefore be used for the unfolding of potential ubiquitinated substrates. This phenomenon will be discussed with reference to the ATPase complex as a molecular chaperone.

1.3.4 Cellular substrates of the 26S proteasome

The association of the 19S regulatory complex with the 20S proteasome confers the requirement for ATP hydrolysis and in most cases ubiquitination of substrates targeted for degradation. There are a large number of substrates whose degradation is known to proceed by this pathway. These include short-lived regulatory molecules such as cyclin B (Murray *et al.*, 1989) and the cyclin-dependent kinase inhibitor p40^{SIC1} (Nugroho and Mendenhall, 1994). Also, the precursor of the NFκB transcriptional activator (Palombella *et al.*, 1994) and its inhibitor IκBα are degraded by this pathway. Degradation of IκBα occurs following phosphorylation on two serine residues (Chen *et al.*, 1995). The tumour-suppressor protein, p53, is known to be degraded largely by this pathway *in vivo* (Chowdhary *et al.*, 1994). In addition, a number of the cellular oncoproteins such as c-Mos (Ishida *et al.*, 1993), c-Jun (Treier *et al.*, 1994), c-Fos (Wang *et al.*, 1996) and c-Erb (Mimnaugh *et al.*, 1996) are also degraded by the ubiquitin proteasome pathway.

1.3.5 Regulation of the 26S proteasome

1.3.5.1 The ‘modulator’

As suggested in section 1.3.2, there are at least two regulators of the activity of the 26S proteasome which are dependent on association of the two sub-complexes. One of these is the 'modulator', a complex which stimulates the proteolytic activity of the 20S proteasome 8-fold (de Martino *et al.*, 1996). It was isolated in a screen to look for activators of proteasome activity. This complex comprises three proteins, of which two, p50-TBP1 and p42-SUG2 are subunits of the 19S regulatory complex. The other protein component, p27, is not a subunit of the 19S complex. Unlike the 19S complex, the 'modulator' has no ATPase activity (Fujiwara *et al.*, 1996), although it may prefer other nucleotides such as GTP or UTP. Hydrolysis of these other nucleotides has been demonstrated for the 26S complex (Hoffman and Rechsteiner, 1996).

The mechanism by which the 'modulator' functions, is unknown. It may help to recruit a 19S complex to either end of the 20S proteasome, or, alternatively, it may form a ternary complex with the 26S complex itself. In this regard, it is similar, but not identical to a second regulator of 26S proteasome activity.

1.4 The AAA family of ATPases

Members of the AAA family (ATPases associated with a variety of cellular activities) represent a set of proteins, from a wide spectrum of organisms, which contain either one or two copies of a highly conserved 230 amino acid ATPase module (Confalonieri and Duguet, 1995). The diversity of function of these proteins suggest an essential but as yet unknown role in many aspects of cellular regulation. Although only a small number of these proteins have been found in prokaryotes, there are 17 in *S.cerevisiae*, belonging to distinct sub-families.

1.4.1 Functions of AAA family members

The FtsH protein of *E.coli*, represents one of the few eubacterial members of this family. This protein functions in the control of cell-division. Homologues of this protein are present as proteases in the inner membrane of mitochondria of

S.cerevisiae, providing further evidence of the prokaryotic origin of this eukaryotic organelle.

The function of members of this family in eukaryotes is less clear. Although six of these ATPases are known to be incorporated into the 26S proteasome, the actual function of these subunits has yet to be elucidated. As mentioned above, three of these family members, Yta10p, Yta11p and Yta12p form proteolytic complexes in mitochondria. Other functions of eukaryotic AAA family members include secretion, vesicle fusion, peroxisome biogenesis and meiosis (Confalonieri and Duguet, 1995).

1.4.2 Macromolecular assemblies of AAA proteins

1.4.2.1 p97/VCP

p97 is an abundant polypeptide found in the nuclear and cytoplasmic compartments of mammalian cells (Peters *et al.*, 1992). As a member of the AAA family, it contains a duplicated ATPase domain. The peptide sequence of p97 is closely related to two other AAA proteins involved in secretion: N-ethyl maleimide sensitive factor (NSF), and its yeast equivalent Sec18p. The primary function of p97 is in the formation of golgi stacks from golgi cisternae, produced after mitosis (Acharya *et al.*, 1995; Rabouille *et al.*, 1995), and in the import of nuclear proteins via its association with the NLS receptor and RanBP1 (Chi *et al.*, 1996; 1997).

When studied by EM, this Mg^{2+} -ATPase appeared as a two-layered hexagonal particle, with a central channel (Harris, 1984). Unlike the eukaryotic 20S proteasome, which has 4 rings of seven subunits arranged around a central pore, this complex is a homo-hexamer, requiring 6 subunits for its ATPase activity (Peters *et al.*, 1992).

1.4.4.2 The *S.cerevisiae* YTA10/ YTA12 proteolytic complex

The Yta10p-Yta12p complex forms a hetero-oligomer of approximately 850kDa (Arlt *et al.*, 1996). Like the 26S complex, the Yta10p-Yta12p protease has both proteolytic and chaperone activity, since a proteolytically inactive complex is still able to mediate assembly of the F₀-F₁ ATP synthase complex (Arlt *et al.*, 1996; Leonhard *et al.*, 1996). In this respect, the proteins which form this complex are different from their bacterial homologue FtsH, which mediates the degradation of the bacterial proteins σ^{32} and SecY (Leonhard *et al.*, 1996) and from Yta11p an AAA protease of the mitochondrial inner membrane space, which, unlike Yta10-12, which has no chaperone activity.

1.4.2.3 Proteasomal ATPases: a ring structure?

EM data (Peters *et al.*, 1992), have been interpreted to suggest that the 19S complex forms a cap on either end of the 20S proteasome. By analogy to the Clp ATPase complexes of prokaryotes, and to the p97 ring in eukaryotes, the ATPases within this 19S complex are thought to adopt a ring conformation (Peters *et al.*, 1992; Dubiel *et al.*, 1993). The advantage of this conformation is obvious, in that it would allow passage of a protein through the regulatory complex into the catalytic chamber below. Observations confirming interactions between adjacent subunits lends weight to this hypothesis (Ohana *et al.*, 1993; Russell *et al.*, 1996; Richmond *et al.*, 1997). The adoption of a ring-like conformation, both in prokaryotes and in eukaryotes, may be a common feature of both chaperone and proteolytic complexes.

1.4.2.3.1 Functions of ATPase assembly

Although the proteasomal ATPases may function to promote 26S complex assembly, they may be also be involved in the unfolding of potential substrates for entry into the 20S catalytic core. In this respect, the ATPases would have a function very similar to

that of the ClpA / ClpX ATP-dependent activators of the bacterial ClpAP and ClpXP proteases. In the absence of ClpP, these two complexes function as chaperones to assist in the disassembly of the Mu transposase tetramer (Levchenko *et al.*, 1995). The binding of these 'anti-chaperones' facilitates the proteolysis of the Mu protein.

Normally, chaperones catalyse the refolding of proteins after stresses such as heat-shock. When this refolding fails, due to the absence of other factors, or because it has taken too long, these chaperones are thought to assist protein degradation by preventing refolding of the protein and targeting it to a proteolytic complex, such as the ClpP protease (Hayes and Dice, 1996).

There is a strong structural similarity between the Clp AP / XP complexes and that potentially formed between the 20S proteasome and the ATPases of the 19S regulatory complex. The Clp AP protease has 2 rings of 7 proteolytic subunits, with a ring of 6 ATPases at either end. In the same way, the 20S proteasome has 7 membered rings of α subunits at each end which may contact the proposed 6 membered ring of ATPases in the 19S complex (Hochstrasser, 1996). In this way, both complexes may fulfil the similar functions of chaperones and proteolytic complexes.

1.5 The *mts2*⁺ gene

The *mts2-1* mutant was isolated in a screen to look for yeast cells which were resistant to the mitotic poison MBC (Gordon *et al.*, 1993). At the restrictive temperature, yeast cells carrying this mutation are defective at the metaphase to anaphase transition. In addition there is a concomitant increase in the intracellular level of high molecular weight ubiquitin conjugates. These results suggested that the protein encoded by *mts2*⁺ was involved in both the control of mitosis and in the ubiquitin pathway for protein degradation.

1.5.1 The product of *mts2*⁺ is a component of the 26S proteasome

Sequence database analysis of the cDNA which rescued the *mts2-1* mutant revealed a 75% identity between its predicted amino acid sequence and that of S4, an ATPase subunit of the human 26S proteasome (Dubiel *et al.*, 1992). Subsequently, it was shown that the human gene could also rescue the *ts* phenotype of the *mts2-1* mutant, as well as a disruption of the *mts2⁺* gene, thereby indicating that S4 and the product of *mts2⁺* were functional homologues, and that Mts2p was likely to be a subunit of the 26S proteasome (Gordon *et al.*, 1993).

Confirmation of the presence of Mts2p in the *S.pombe* 26S proteasome was provided by western blotting of purified wild type 26S proteasome from *S.pombe*, with an antibody raised against recombinant Mts2 protein (Seeger *et al.*, 1996). Moreover, when purified 26S proteasome preparations from the *mts2-1* mutant which had been grown at the restrictive temperature and blotted using the same antibody, the mutant protein was present (Seeger *et al.*, 1996). This mutant proteasome was *ts* for ubiquitin conjugate degradation activity. By contrast, the mutant Mts3 protein, was not integrated into the proteasome. Purified proteasome preparations from both *mts2-1* and *mts3-1* mutants could still support the hydrolysis of small fluorogenic peptides, but not that of ubiquitin conjugates, indicating that both the Mts2 and Mts3 proteins were essential for ubiquitin-conjugate degradation

Further evidence for the association of Mts2p with the 26S proteasome was obtained during a screen to look for murine multicopy suppressors of *mts2-1*. One suppressor was identified as the murine homologue of the mammalian suppressor of *sgv1*, MSS1 (Gordon *et al.*, 1993). Like *mts2⁺*, MSS1 encoded a subunit, S7, of the 26S proteasome (Dubiel *et al.*, 1993). Unlike S4, overexpression of mouse MSS1 could not rescue the *mts2* null allele. This result suggested that suppression by MSS1 required the product of the *mts2-1* allele.

1.5.2 Involvement of Mts2p in the metaphase anaphase transition

At mitosis, cells which have completely replicated their DNA, and which have satisfied all of the checkpoint criteria (Hartwell and Weinert, 1989), proceed to M-phase

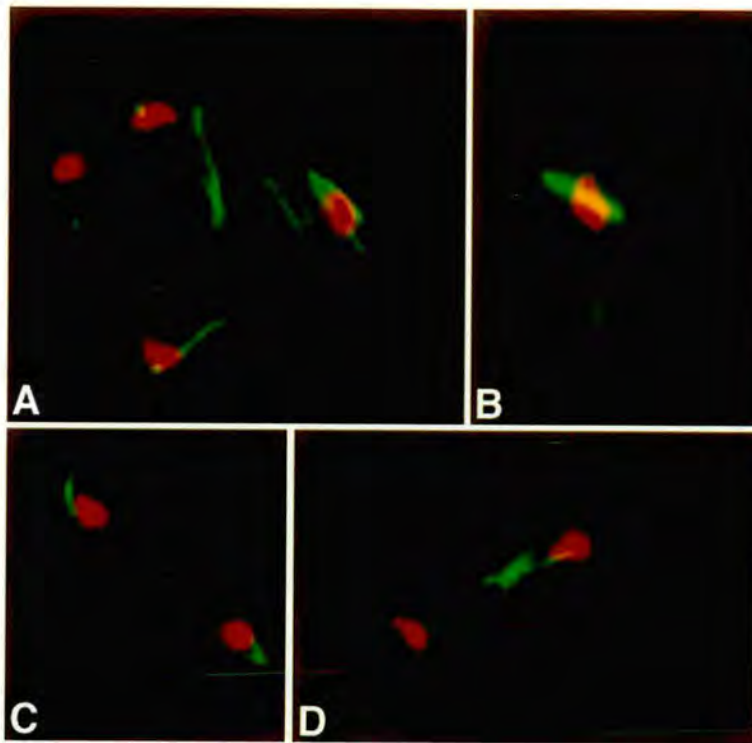


Figure 1.4 Cytology of *S.pombe* cells during mitosis. (taken from Gordon *et al* 1996)

Wild type cells were stained with the anti-tubulin antibody TAT1 (shown as green) and DAPI (shown as red). The two images were merged on the computer to give the image shown. The DAPI signal was changed on the computer from blue to red to increase the resolution of the desired structures. A, interphase cells; B, mitotic metaphase cell; C, late anaphase cell; D, postanaphase.

of the cell cycle, where newly replicated chromosomes are equally partitioned into two daughter cells. The cytological events which take place in a wild type fission yeast cell during mitosis are shown in Figure 1.4.

The metaphase defect of the *mts2-1* mutant was the first *in vivo* evidence that Mts2p and the 26S proteasome, were involved in the control of this stage of mitosis (Gordon *et al.*, 1993). A similar phenotype was observed in two *S.cerevisiae* mutants, *cim3-1* and *cim5-1* (Ghislain *et al.*, 1993). Like Mts2p, the products of the *CIM3* and *CIM5* genes are both ATPase subunits of the 26S proteasome, and are homologous to S8 and S7 respectively (Dubiel *et al.*, 1995). The common phenotype observed in these mutants suggested that the proteasome was responsible for two main functions during mitosis: degradation of cyclin B, which prevented exit from mitosis, and the degradation of a factor which held sister chromatids together until anaphase.

1.5.2.1 Cyclin B destruction during mitosis

It was established that cyclin B destruction was not required for sister chromatid segregation (SCS) but for exit from mitosis, and that both processes required the same degradation machinery, which involved the ubiquitin pathway (Murray and Kirschner, 1989; Glotzer *et al.*, 1991; Holloway *et al.*, 1993; Surana *et al.*, 1993). Fractionation experiments using cyclin B from clams showed that cyclin ubiquitination required components present in 4 different fractions. Only one of these, a component which ligated the ubiquitin to the cyclin molecule, was cell cycle regulated (Hershko *et al.*, 1994; Sudakin *et al.*, 1995). This ubiquitin ligase activity was attributed to a multi-subunit particle called the cyclosome (Sudakin *et al.*, 1995).

Separate experiments to look for mutations which prevented degradation of cyclin B in budding yeast enabled the isolation of three genes *CDC16*, *CDC23* and *CDC27*. Mutations in these genes resulted in a metaphase arrest indicating that in addition to preventing cyclin B destruction, they were also required at an earlier stage in mitosis. The products of these genes were subsequently shown to interact as part of a multi-subunit complex which was required for the ligation of ubiquitin to cyclin (Lamb *et*

al., 1994; Tugendreich *et al.*, 1995; Irniger *et al.*, 1995; King *et al.*, 1995). This anaphase promoting complex (APC), the yeast equivalent of the cyclosome, was specific for the degradation of mitotic cyclin B. Other cyclins such as the budding yeast Clb5 protein are degraded in an APC-independent manner (King *et al.*, 1995)

Homologues of the components of this complex have been found in other organisms. In fission yeast, the *nuc2⁺* and *cut9⁺* genes are the homologues of *CDC27* and *CDC16* respectively (Hirano *et al.*, 1988; Samejima and Yanagida, 1994; Peters *et al.*, 1996). In *Xenopus* the APC has been shown to comprise at least 8 subunits, of which 4 have been microsequenced. Three were found to have a strong homology to the previously identified budding yeast genes, and a fourth, APC1, was homologous to the *Aspergillus nidulans* BimE gene and the *S.pombe cut4⁺* gene (Peters *et al.*, 1996).

1.5.2.2 Control of sister-chromatid separation

The failure of *mts2-1* cells to proceed through this metaphase arrest, coupled to the presence of Mts2p in the proteasome suggested that one function of the proteasome was to degrade some unknown protein factor to enable sister chromatid separation (SCS) to take place. Experiments using a non-degradable form of cyclin B demonstrated that degradation of this molecule was not required for SCS, prompting speculation of a 'molecular glue' holding sister chromatids together until anaphase (Holloway *et al.*, 1993; Surana *et al.*, 1993).

A number of candidates for this 'glue' have been proposed both in yeast and in *Drosophila*. In fission yeast, the product of the *cut2⁺* gene (Funabiki *et al.*, 1996b) was found to restore proper SCS. However, while overexpression of *cut2⁺* resulted in a metaphase arrest with unsegregated daughter nuclei (Funabiki *et al.*, 1996b), a deletion of *cut2⁺* resulted in the same phenotype. One interpretation of these results is that instead of being a molecular glue, the product of *cut2⁺* probably acts as a regulator of SCS.

The product of the budding yeast *PDS1* gene was also proposed as a candidate for holding sister-chromatids together (Cohen-Fix *et al.*, 1996). Despite being ubiquitinated and degraded at anaphase in an APC- and D-box-dependent manner, deletion of *PDS1* only resulted in 50% of the cells bypassing an anaphase block imposed by a mutation in a gene encoding a subunit of the APC. These experiments suggest that the product of *PDS1* may be a regulator of APC activity rather than the 'glue' itself.

Regulators of SCS have also been found in *Drosophila melanogaster*. Mutations in three genes *fizzy* (Sigrist *et al.*, 1995; Philip and Glover, 1997), *threerows* and *pimples* (Stratmann and Lehner, 1995). have been shown to result in defective SCS. In the case of *fizzy*, a homologue of the budding yeast *CDC20* gene (Sigrist *et al.*, 1995), mutations also prevent the sequential degradation of cyclins A, B and B3 suggesting that in some way, the product of this gene interacts with the APC. By contrast, mutations in the *threerows* and *pimples* genes do not block SCS, but prevent the degradation of cyclin B.

As yet, the molecules which directly mediate sister-chromatid cohesion have not been isolated. Although it is possible that the degradation of only one gene product is required for SCS, it is likely that a number of gene products, such as Cut1p and Cut2p, regulate this process.

1.6 Project aims

1.6.1 The isolation of genes which interact with *mts2*⁺

One method for the isolation of interacting genes involves the isolation of multicopy suppressors of the conditional lethal *mts2-1* mutation. Transformation of the *mts2-1* mutant with an *S.pombe* cDNA library which has been cloned into a vector under the control of a strong promoter, should facilitate the isolation of both the endogenous gene as well as other genes which can suppress this mutation. It might be expected that one multicopy suppressor of the *mts2-1* mutant will be the *S.pombe* homologue

of the mouse MSS1 gene. This screen may also enable isolation of genes encoding other subunits of the 26S proteasome. The vector of choice, in this case, is a derivative of pREP1, which contains the thiamine repressible *nmt1* promoter. Genes cloned under the control of this promoter are expressed at a high level (see Table 2.1) in the absence of thiamine (Maundrell, 1990).

A second and more classical genetic approach to the isolation of suppressors, is to look for revertants of the original conditional lethal mutation. Revertants can be generated either spontaneously, or by the use of mutagenic agents such as UV light. This method of suppression involves the alteration of an interacting protein, which compensates for the alteration in the mutant Mts2 protein.

Intragenic suppressors generated by these means could either occur as a result of a reversion of the original mutation, or by second site suppression within the *mts2* gene. Extragenic suppressors isolated by this method are almost always in genes whose products are known to interact physically with the original gene products (Jarvik and Botstein, 1975). Although this technique is more likely to generate extragenic suppressors of the *mts2-1* mutation, it relies on the extragenic suppressor mutation also being conditional. In this way, the suppressor could be crossed away from the original *mts2-1* mutation and characterised by standard molecular genetic techniques.

The genetic isolation of suppressors has proved very useful for the isolation of proteins which interact in complexes. Spontaneous reversion (pseudoreversion analysis) of a mutation in the *S.cerevisiae act1* gene, encoding actin, led to the isolation of components of the cytoskeleton (Novick *et al.*, 1989). In the same way, it is hoped that this type of analysis will lead to the isolation of genes encoding other subunits of the 26S proteasome.

A more recent screen will also be used to look for proteins which interact with the product of the *mts2⁺* gene. The yeast 2-hybrid system provides a means to look for protein-protein interactions *in vitro* (Fields and Song, 1989). This system relies on the reconstitution of a functional transcriptional activator from the *S.cerevisiae* Gal4p



DNA-binding domain and activation domain, which are encoded on separate plasmids. The interaction of two gene products, encoded on these two plasmids, results in expression of the β -galactosidase gene, which is under the control of the Gal4 promoter, thereby providing a means of selecting for an interaction between two gene products. The use of an *S.pombe* cDNA library, cloned into the appropriate vector, in this system, may help to isolate interactions between the product of *mts2*⁺ and other subunits of the 26S proteasome.

1.6.2 Analysis of mutations in the *mts2* gene

The original MBC^R screen for *mts* mutants facilitated the isolation of three *ts* alleles of the *mts2* gene (C.Gordon, unpublished results). A second independent screen, to look for enhancers of position effect variegation in *S.pombe* led to the isolation of 3 cold-sensitive (*cs*) mutants in three genes *cep1*⁺-*cep3*⁺ (J-P. Javerzat, unpublished results). Over-expression of the *mts2*⁺ cDNA rescued a disruption of the *cep2*⁺ gene indicating that *cep2*⁺ was *mts2*⁺. Four alleles of the *cep2* mutant were isolated. In total, then the two screens have enabled the isolation of 7 conditional *mts2* alleles.

The molecular characterisation and analysis of the phenotypic effect of these mutations in the *mts2* gene provides an opportunity to study the differences in phenotype resulting from independent mutations. This study may help to identify essential amino acid residues within Mts2p and, by comparison, in the other members of the proteasomal AAA family.

Chapter 2

CHAPTER 2 Materials and Methods

2.1 Commonly used reagents and buffers

Most of the methods used in this work are described (Sambrook *et al.*, 1989). All chemicals were of analytical grade and were bought from Sigma, British Drug Houses (BDH), Gibco-BRL, Fisons or Pharmacia. H₂O refers to distilled autoclaved water. Eppendorf tube refers to a 1.5ml centrifuge tube, except where stated. High speed centrifugation in eppendorf tubes refers to a bench-top eppendorf centrifuge 5415C at 14000rpm.

Tris.Cl

Tris base (tris[hydroxymethyl]aminomethane, Sigma T-1503) was dissolved in H₂O and the pH adjusted to the required value by the addition of HCl.

EDTA

A stock solution of 0.5M EDTA (ethylenediaminetetra-acetic acid di-sodium salt, Fisons D-0452) was made by dissolving solid EDTA in H₂O, adjusting the pH to 8.0 with NaOH and adding water to the required volume.

TE

A buffer solution comprising 10mM Tris.Cl (pH7.5) and 1mM EDTA.

Phenol

For work with DNA and proteins, phenol (Fisons, P-2360) was pre-equilibrated with 1M Tris.Cl (pH7.5), followed by equilibration with TE buffer. In order to retard oxidation of the phenol solution, 0.1% (w/v) hydroxyquinoline (Sigma, H-5876) was added. The equilibrated phenol was stored at 4°C in the dark.

Chloroform

Chloroform (BDH 10077) refers to a 24:1 (V/V) mixture of chloroform and isoamyl alcohol (Sigma, I-1381).

Sodium acetate

Sodium acetate was dissolved in H₂O, the pH adjusted to 5.2 with acetic acid and H₂O added to a final concentration of 3M.

Ethidium bromide

Ethidium bromide (Sigma, E-875) was dissolved as a stock solution of 10mg ml⁻¹ in H₂O and stored at 4°C in the dark. For use in agarose gels, 5µl was added to 100ml of molten agarose.

Loading buffer

A 10x stock of loading buffer for gel electrophoresis of nucleic acids was prepared and stored at room temperature:

40% glycerol
1% (w/v) orange G (Sigma, O-1625)
10mM EDTA

TBE

TBE was made up as a 20x stock, autoclaved and stored at room temperature:

<u>stock</u>		
1.78M Tris base		216g
1.78M boric acid		110g
20mM EDTA	0.5M	40ml
H ₂ O		to 1 litre

TAE

TAE was made up as a 20x stock solution and stored at room temperature:

<u>stock</u>	
0.8M Tris base	96.4g
HOAc	22.8ml
0.5M EDTA	40ml

2.2 Nucleic acid manipulations

2.2.1 Dissolving and storage

All DNA was dissolved in H₂O and stored at -20°C.

2.2.2 Extraction with phenol-chloroform

Proteins were removed from solutions containing DNA and RNA by extraction with an equal volume of a 1:1 mixture of phenol and chloroform. Traces of phenol were removed by a extraction with chloroform. Extractions were carried out by adding a volume of phenol-chloroform equal to that of the nucleic acid solution. The two solutions were mixed thoroughly by vortexing to form an emulsion and then separated by high speed centrifugation in a microfuge for 2 minutes at room temperature. The aqueous phase was then transferred to a fresh tube, carefully avoiding protein at the interface of the two phases, and the nucleic acids recovered by precipitation.

2.2.3 Precipitation of nucleic acids

DNA was precipitated by the addition of 1/10th volume of 3M sodium acetate (pH5.2) and 2 volumes of ice cold ethanol. The solution was mixed thoroughly by vortexing, cooled at -70°C for 15 minutes and the nucleic acids pelleted by centrifugation for 15 minutes at 4°C. Following precipitation, salt was removed by washing the nucleic acid pellet in 100µl of 70% (v/v) ethanol. The pellet was air dried and then redissolved in H₂O.

2.2.4 Quantification of nucleic acids

DNA prepared by bulk methods was quantified by spectrophotometry. This involved taking an absorbance reading at a wavelength of 260nm. An A₂₆₀ of 1.0 corresponds

to approximately $50\mu\text{g ml}^{-1}$ for double-stranded DNA, $40\mu\text{g ml}^{-1}$ for RNA and $33\mu\text{g ml}^{-1}$ for single-stranded DNA such as oligonucleotides. DNA prepared by polymerase chain reaction (PCR) or by purification following restriction enzyme digestion can be quantified by ethidium bromide staining after agarose gel electrophoresis. Ethidium bromide binds proportionately to DNA by intercalation, and fluoresces under UV light. The nucleic acid sample is run through an agarose gel containing ethidium bromide at $0.5\mu\text{g ml}^{-1}$, in parallel with a nucleic acid of known concentration (usually molecular weight markers), and visualised on a UV trans-illuminator. The amount of DNA present can be calculated from the relative staining intensities of the bands.

2.2.5 Plasmid vectors

pREP1/pREP41/pREP81/pREP3X

These constitute a family of yeast shuttle vectors based on the thiamine repressible *nmt1* promoter from fission yeast (Maundrell, 1990). The promoter and polyadenylation signal of the *nmt1* gene are used to permit thiamine-mediated control of transcription of cloned genes (Maundrell, 1993). These vectors contain the *S.cerevisiae* *LEU2* gene and the *S.pombe* *ars* origin of replication (Figure 2.2). They are maintained extrachromosomally at a high copy number. The polylinker contains *NdeI*, *SalI*, *BamHI* and *SmaI*. In minimal medium containing no exogenous thiamine, the *nmt1* promoter is fully de-repressed allowing high levels of expression of cloned genes. Thiamine added to the medium is sequestered in the cells resulting in a rapid rise in the intracellular thiamine concentration and concomitant repression of *nmt1* transcription (Tommasino and Maundrell, 1991). Despite repression in the presence of $4\mu\text{M}$ thiamine, there is still some residual activity from this promoter (Maundrell, 1993). The pREP41 and pREP81 vectors are identical to pREP1 except that they carry TATA box mutations which result in lower levels of expression from the *nmt1* promoter (Basi *et al.*, 1993) (see Table 2.1). pREP3X was used in the construction of the cDNA libraries used in this work, and was a gift from Chris Norbury.

pUC based plasmids

The pUC-based plasmids are used for the manipulation of foreign genes in *E.coli* (Vieira and Messing, 1982). They contain the pBR322 derived ampicillin resistance

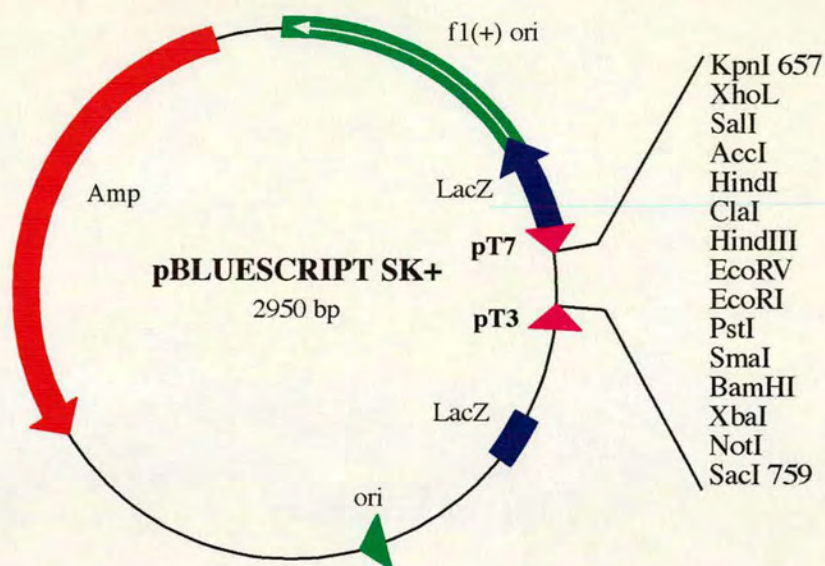


Figure 2.1 General cloning vector pBLUESCRIPT (pSKII+)

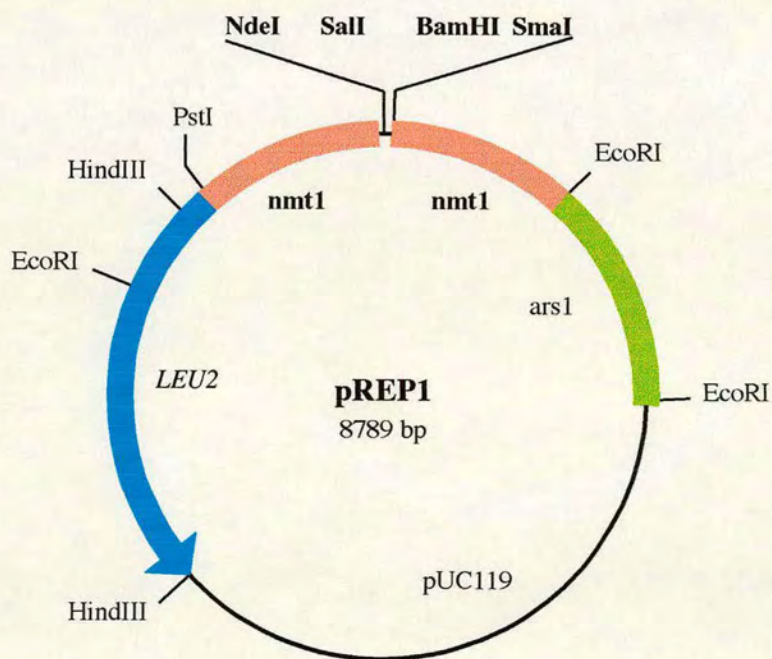


Figure 2.2 The *S.pombe* expression vector pREP1 (41, 81)

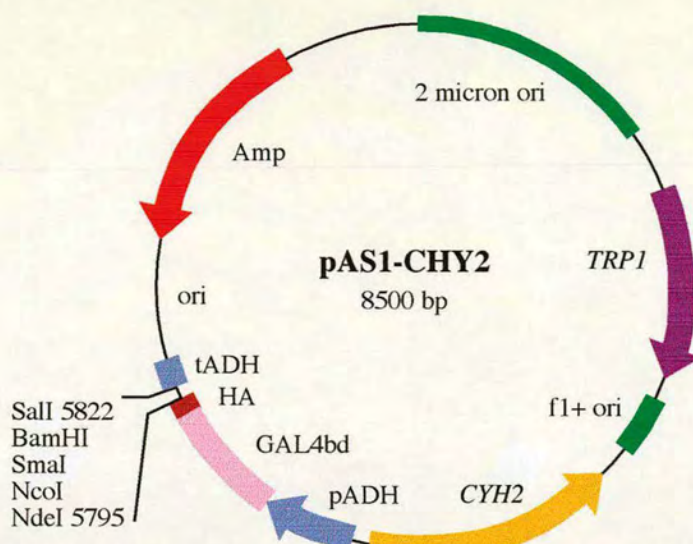


Figure 2.3 2-hybrid 'bait' plasmid pAS1-CYH2

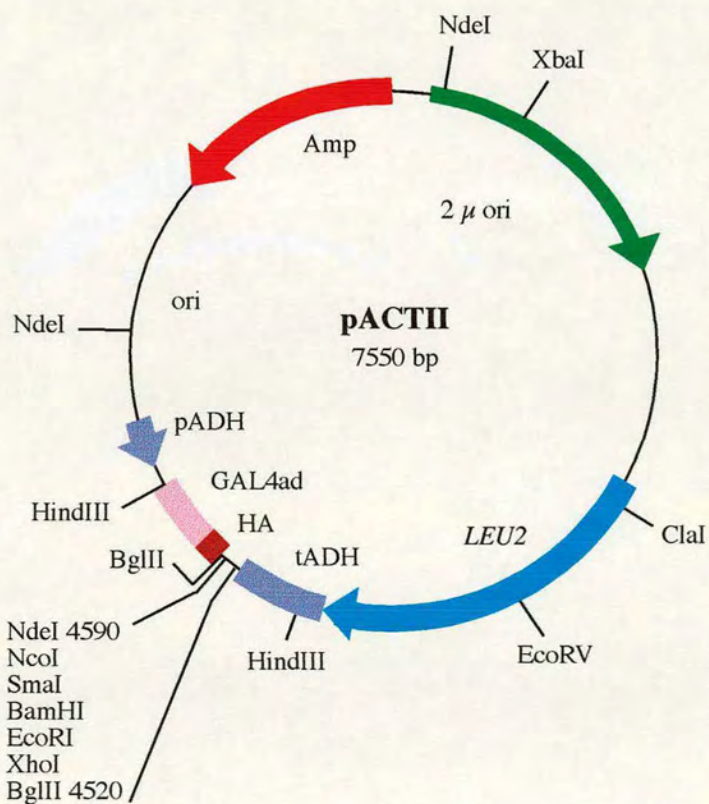


Figure 2.4 2-hybrid 'prey' plasmid pACTII

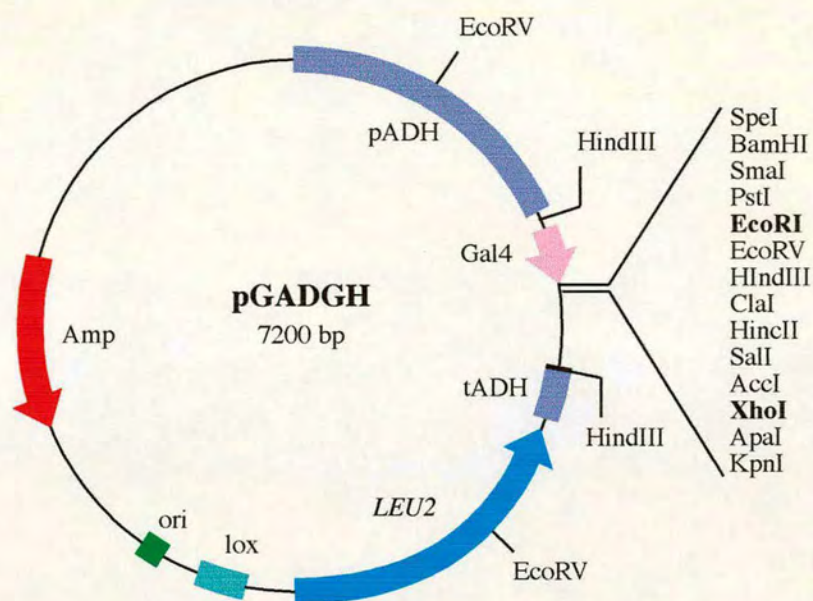


Figure 2.5 2-hybrid library 'prey' plasmid pGADGH

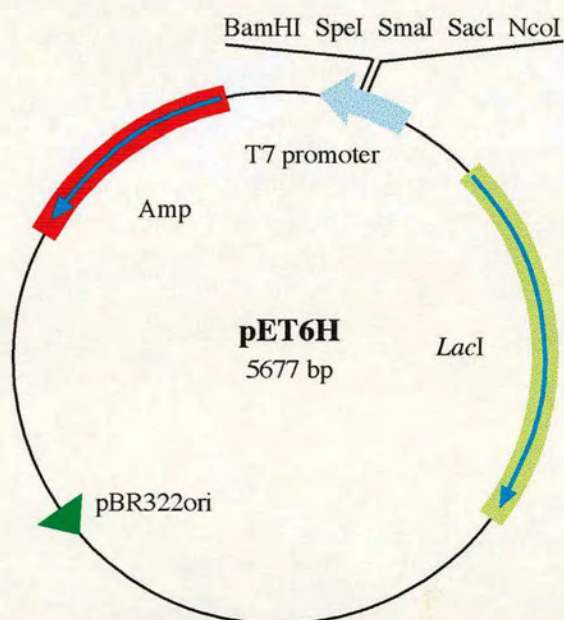


Figure 2.6 pET6H, used for expressing fusion proteins *in vitro*

gene and origin of replication and a polylinker inserted into the *E.coli lacZ* gene. Non-recombinant plasmids are able to synthesise the enzyme β -galactosidase which breaks down the X-gal to release a blue pigmented derivative. In recombinant plasmids the *lacZ* gene is interrupted by foreign DNA resulting in a failure to produce this blue pigment, resulting in white colonies. The vector pBluescript KS⁺ (pKS⁺) (Stratagene, 212208) is a pUC based plasmid and was used extensively throughout this project (see Figure 2.1).

TABLE 2.1 (adapted from Basi *et al.*, 1993)

Characteristics of the pREP1, pREP41 and pREP81 vectors and their relative promoter strengths under inducing and repressing conditions

Vector	TATA box	Relative promoter activity ¹	
		-thiamine	+thiamine ²
pREP1	ATATATAAA	80	1
pREP41	ATAAA	12	0.06
pREP81	AT	1	0.004

¹ Relative promoter activity was measured as chloramphenicol acyl transferase (CAT) activity.

² Thiamine was prepared as a 20mM stock in H₂O and stored in the dark. It was added to a final concentration of 4 μ M.

pASI-CYH (pAS2)

This vector (Figure 2.3) was used extensively for work with the yeast 2-hybrid system (Fields and Song, 1989). It contains the N-terminal (amino acids 1-147) DNA-binding region of the *S.cerevisiae* GAL4 transcription factor fused to the haemagglutinin (HA) epitope under the control of the *ADH* promoter. In addition it contains the *TRP1* gene as an auxotrophic marker, and the *CYH2* gene which confers susceptibility to the protein synthesis inhibitor cycloheximide. Genes can be cloned in frame with the HA epitope and expressed as fusion proteins. Fusion proteins can be detected on western blots using the 12CA5 anti-HA antibody (Boehringer).

pACTII

pACTII was also used for the 2-hybrid system. This vector (Figure 2.4) contains the *S.cerevisiae* GAL4 activation domain (amino acids 768-881) expressed from the *ADH* promoter and fused to the HA epitope. Genes can be cloned in frame downstream of this epitope. Instead of the *TRP1* gene, pACTII contains the *LEU2* marker.

pGADGH

An *S.pombe* cDNA library, obtained from D. Beach, had been cloned into this vector (Figure 2.5). Like pACTII, it contains the sequences for the GAL4 activation domain (amino acids 768-881) and the *LEU2* nutritional marker. However, there is no HA epitope fused downstream of the activation domain. It has an extensive multiple cloning site (MCS) with many unique restriction sites. Fusion proteins can be expressed at high levels from the constitutive *ADH* promoter. In addition, the fusion protein is targeted to the yeast nucleus by nuclear localisation sequences that have been added to the activation domain sequence.

pET6H

This vector (Figure 2.6) is derived from the expression vector pET11D and contains 6 copies of the histidine codon which, when fused in frame to the gene of interest and expressed in bacteria, results in the addition of a histidine tag. This can then be used for purification of the protein through a nickel agarose column. This vector was used for the expression of recombinant yeast proteins (see Chapter 4).

2.3 Molecular analysis of nucleic acids

2.3.1 Restriction enzyme analysis

Restriction enzymes were used to cut DNA according to the manufacturer's instructions. The Boehringer restriction enzyme buffer system was used. Routinely, 1-5µg of DNA was incubated with 10 Units of restriction enzyme and the appropriate buffer in a final volume of 20µl at 37°C for 1-15 hours.

2.3.2 Dephosphorylation of DNA

In order to prevent the self-ligation of vectors, the 5' phosphate was removed by alkaline phosphatase treatment. Linearised vector DNA was cleaned by extraction with phenol-chloroform followed by ethanol precipitation. The pellet was resuspended in TE and treated with calf intestinal alkaline phosphatase (CIP) (Boehringer) according to the manufacturer's instructions.

2.3.3 Ligations

Ligation of DNA with cohesive termini was performed using T4 DNA ligase (Boehringer 862509) and ligation buffer. Routinely between 100ng-1 μ g DNA was ligated with 1unit of ligase (1unit/ μ l) in a total volume of 20 μ l. Ligation reactions were incubated for 16hrs at room temperature. Ligation of blunt-ended termini was achieved by incubation with a ten-fold excess of DNA ligase and was carried out at 16°C.

2.3.4 Agarose gel electrophoresis of DNA

Electrophoresis of DNA through 0.8%-1.2% agarose gels was used to separate DNA fragments ranging in size from 100bp to 20kb. The appropriate amount of agarose was dissolved in 1xTAE or TBE buffer. Once the gel had cooled sufficiently, ethidium bromide was added to a final concentration of 0.5 μ g ml⁻¹. The length of time for which the gel was run was dependent on the size of the DNA fragments to be separated. Size markers were used on all gels and were either bacteriophage lambda DNA digested with *Hind*III, ϕ X174 DNA digested with *Hae*III or a 1kb ladder (Gibco-BRL, 5615SA).

2.3.5 Purification of DNA fragments

DNA fragments of between 300bp and 3kb were purified from agarose gels by means of two commercially available kits: Wizard Clean-up kit (Promega) and QIAquick gel extraction kit (Quiagen). Manufacturer's instructions were followed routinely.

2.4 Radiolabelling of DNA fragments

Radio-labelled fragments of DNA were used as probes for Southern blotting (section 2.5).

2.4.1 Random prime labelling

This is based on the method described by Feinberg and Vogelstein (1983), and uses random hexanucleotides which bind to the DNA fragment and act as primers for polymerisation by the Klenow fragment of DNA polymerase I. This guarantees an equal degree of labelling along the entire length of the template DNA. The constituents of this reaction are commercially available as a kit which contains dNTPs, "reaction mixture," and Klenow enzyme (Boehringer). The DNA (20-50ng in 10 μ l H₂O) was denatured by boiling for 5 minutes, cooled rapidly on ice to prevent re-annealing and 2 μ l reaction mix and 1 μ l of spermidine added. The following dNTPs were then added (all cold dNTPs at 0.5mM): 3 μ l [α ³²P]dCTP +1 μ l each of dATP, dGTP, dTTP. After the addition of 1 μ l Klenow polymerase (2U/ μ l), the labelling reaction was incubated at 37°C for 1hr.

2.4.2 Estimation of label incorporation into radiolabelled probes

The proportion of radiolabelled dNTPs incorporated into labelled DNA was measured by comparing the counts per minute (CPM) before and after removal of unincorporated label. 1 μ l labelled probe was spotted onto a Whatman GF/B glass-fibre filter disc, and the labelled DNA precipitated onto the disc by the passage of 10ml 5% TCA sucked through the disc under vacuum.

2.4.3 Removal of unincorporated label

Labelled probe was separated from unincorporated dNTPs by gravity elution through a sepharose column. A Sephadex G10 Nick column (Pharmacia) was equilibrated by addition of 6ml TE (pH7.5). To the labelled probe 400 μ l TE was added and this was loaded onto the column. Following elution of the first 400 μ l from the column, the

subsequent two 400µl fractions were collected. The peak of radioactivity, containing the labelled probe, was found in the first of these fractions.

2.5 Southern blotting

The hybridisation of radiolabelled nucleic acids to DNA immobilised on a nitro-cellulose or nylon filter (Hybond N+, Amersham RPN 203B) is known as Southern blotting (Southern, 1975).

2.5.1 Transfer

DNA digested with the appropriate restriction enzymes was separated on an agarose gel. Once migration was judged by UV to have proceeded to the correct point, the DNA was denatured by immersion and agitation in 0.4M NaOH/1.5M NaCl for 30 minutes followed by neutralisation in 0.5M Tris.HCl (pH7.5)/3M NaCl for 30 minutes. After rinsing the gel in 2xSSC the gel was transferred to a Southern blotting tank with 20xSSC in the reservoir and Whatman 3MM chromatography paper as a wick. After covering the rest of the exposed wick with clingfilm to reduce evaporation, the gel was overlaid with a piece of membrane which had been soaked in the 2xSSC. Air bubbles, which could prevent efficient transfer of the DNA onto the membrane, were removed by rolling a 10ml pipette firmly over the membrane. Following this, 4 pieces of 3MM paper which had been soaked in 2xSSC were placed on top of the membrane. On top of this was placed a stack of paper towels and a heavy weight. Blotting was allowed to proceed overnight. Following transfer, DNA was permanently attached to the membrane by exposure to 150 mJ of UV irradiation (254nm) using a BioRad GS gene linker.

2.5.2 Hybridisation

Filters were pre-hybridised in 40ml of ¹hybridisation solution* in a Techne hybridisation oven for 1-2hrs. The radiolabelled probe was then denatured by boiling for 10 minutes, cooled rapidly on ice and added to the hybridisation solution. Hybridisations

were carried out at 65°C overnight. Filters were washed x3 in 0.2xSSC/0.1% SDS at 65°C then sealed in clingfilm before autoradiography.

¹ Hybridisation solution

	<u>stock</u>	<u>/litre</u>
NaPPi		1g
SDS		1g
BSA		0.5g
Ficoll		0.5g
PVP		0.5g
SSC	20X	250ml
H ₂ O		744ml

* Prior to pre-hybridisation of filters, denatured salmon sperm DNA, diluted to 10mg ml⁻¹ in H₂O, was added to a final concentration of 75µg ml⁻¹. In addition a further 0.8ml of 10% SDS was added to 40ml of pre-hybridisation solution.

2.5.3 Autoradiography

Autoradiography of ³²P-labelled DNA was carried out using Kodak X-omat AR film at -70°C in cassettes containing tungsten intensifying screens. For ³⁵S autoradiography, as used in sequencing gels, the cassettes were left at room temperature. Films were developed using a Fuji automatic processor.

2.5.4 Phosphoimaging

Use of a Molecular Dynamics phosphorimager enabled the visualisation of radiolabelled bands by exposure of the hybridised filter in a phosphoimaging cassette followed by laser scanning using Image Quant software. This method substantially reduces the time taken for development of weak signals.

2.5.5 Stripping

Filters to be re-probed were stripped by placing them in a solution of boiling 0.5% SDS. The solution was removed from the heat and gently shaken at room temperature until the solution had cooled.

2.6 Polymerase Chain Reaction

2.6.1 General PCR methodology

The polymerase chain reaction (PCR) is a technique for the amplification of DNA fragments from genomic or plasmid DNA. Amplification was performed for 35 cycles using 100ng of chromosomal DNA or 1-10ng of plasmid DNA with the Promega *Taq* polymerase and buffer system (M1861) in a Hybaid omnigene machine. Programmes were as follows:

94°C	3 minutes	(initial denaturation)	1 cycle
51°C*	30 seconds	(annealing)	
72°C	1-2 minutes	(elongation)	35 cycles
92°C	30 seconds	(denaturation)	
51°C*	30 seconds	(annealing)	1 cycle
72°C	5 minutes	(final annealing)	

*- Annealing temperatures varied depending upon the melting temperatures (T_m) of the primers. Details of all primers used throughout this project and the conditions for each individual reaction are shown (Appendix A).

Reaction mixtures were as follows:

Template DNA	1-100ng
¹ Primer 1	1µl of 100ng/µl

Primer 2	1µl of 100ng/µl
10x reaction buffer	5µl
MgCl ₂ (25mM)	4µl
dNTPs (10mM)	2µl
<i>Taq.</i> polymerase	0.5µl
H ₂ O	to 50µl

Each reaction was overlaid with 50µl of PCR grade mineral oil (Sigma, M-3516) to prevent evaporation.

¹Oligonucleotides used as primers in PCR reactions were synthesised by the MRC oligonucleotide synthesis service. Prior to use they were purified from ammonium stock, eluted in water and the DNA concentration measured by spectrophotometry (section 2.2.4).

2.6.2 PCR from bacterial colonies

This was based on the method of Maskell *et al.*, (1993) and involved the boiling of a bacterial colony in 50µl H₂O for 5 minutes. 10µl of the supernatant was then used in a 100µl PCR reaction.

2.6.3 PCR from yeast patches

The procedure, based on the method of Ling *et al.*, (1995), was used. Zymolyase was utilised as a means of degrading the cell wall of the yeast cells, which more widely used methods failed to do. The colony or patch is touched with the tip of a disposable pipette and rinsed in 10µl of incubation solution: 1.2M sorbitol, 100mM NaPO₄ (pH7.4), 2.5mg/ml⁻¹ Zymolyase. This is incubated at 37°C for 5 minutes after which 5µl of this mix is used in a 100µl PCR reaction.

2.7 DNA sequencing

DNA sequencing was carried out using the chain termination method (Sanger *et al.*, 1977). Two methods were used for the sequencing of double stranded plasmid DNA and DNA amplified by PCR, and throughout this project, 2 different labels were used:-

1. ^{35}S dATP
2. Fluorescent dyes

2.7.1 ^{35}S dATP sequencing

This was performed on two types of template:

1. Double-stranded plasmid DNA
2. PCR-generated double-stranded DNA fragments

2.7.1.1 Sequencing of double-stranded plasmid DNA

This was carried out using the Sequenase version 2.0 kit (USB). 4 μg of plasmid DNA in 20 μl of H_2O was denatured by adding an equal volume of 0.2M NaOH/0.02M EDTA (pH8.0) and incubating at 37°C for 5 minutes. The DNA was precipitated by the addition of 4 μl of 3M NaOAc (pH 5.2) and 80 μl of ice cold absolute ethanol followed by cooling at -70°C for 15 minutes and centrifugation at 4°C for 15 minutes. The pellet was washed with 70% ethanol, air dried and resuspended in 7 μl H_2O . To this was added 2 μl of 5x Sequenase buffer and 1 μl of primer (10ng/ μl). After incubation at 37°C for 10 minutes, the following was added:

0.1M DTT	1 μl
$^1[\alpha\text{-}^{35}\text{S}]$ dATP	0.5 μl
labelling mix	2 μl (USB kit stock diluted 1:8 in H_2O)
T7 polymerase	2 μl (diluted to 1.5U/ μl in dilution buffer)

$^1 [\alpha\text{-}^{35}\text{S}]$ dATP: Amersham 37.0 Mbq 1.00mCi; 10 μCi / μl

The mixture was incubated at room temperature for 5 minutes. 3.5µl was then added to 2.5µl of each termination mix (pre-warmed at 37°C) and incubated at 37°C for 5 minutes. 4µl of formaldehyde stop mix was added and the samples left on ice until ready to run on the gel.

2.7.1.2 Sequencing of PCR-generated double-stranded DNA

This method was adapted from Winship *et al.*, (1989) and the standard Sequenase protocol. It allows direct sequencing of PCR amplified DNA by using one of the pair of primers, used for the PCR reaction, as a primer for the sequencing reaction. The appropriate PCR reaction was carried out to generate sufficient DNA (1µg/reaction). The reactions were purified as described (section 2.3.5). The DNA was resuspended in 6µl of H₂O. To the DNA was added 1µl DMSO, 1µl primer (3.2pmol/µl) and 2µl 5x Sequenase buffer (200mM Tris.Cl pH7.5, 100mM MgCl₂, 250mM NaCl). The mixture was boiled for 3 minutes and snap-frozen in liquid N₂. The 4 termination mixes were prepared by adding 0.5µl DMSO to 2µl of each ddNTP mix in separate eppendorf tubes. The labelling mix was prepared by the addition of 1µl 0.1M DTT, 2µl labelling mix (7.5µM dCTP, dGTP and dTTP), 1µl α-[³⁵S] dATP and 3 Units T₇ DNA polymerase to the denatured DNA as it was thawing from the liquid N₂. The reaction was mixed and immediately evenly divided between the four termination mixes. Following incubation of the termination mixes at 37°C for 5 minutes, 4µl of formamide 'Stop' solution was added and the reactions were left on ice or stored at -20°C until required.

2.7.1.3 Polyacrylamide gel electrophoresis of ³⁵S labelled DNA samples

This was carried out using the BRL sequencing system. The plates were cleaned thoroughly with a mild detergent, rinsed with distilled water and wiped with 70% ethanol. One of the plates was treated with di-chloro di-methylsilane (BDH, 63216 4J) to facilitate easy removal of the gel. Spacers were inserted between the plates and the apparatus was clamped and laid almost horizontally.

The gel was made by measuring 100ml sequencing acrylamide (40%: 0.8% mono:bis-acrylamide) (National Diagnostics, 13456) and adding 0.5ml freshly made 10% Ammonium persulphate (Sigma, A-9164) and 50µl of TEMED (N,N,N',N' - tetramethylethylenediamine (Sigma, T-8133) and mixed. The rapidly polymerising solution was poured into the gap between the plates until the acrylamide began to drip from the bottom. At this point the gel was laid flat, the comb inserted to form a loading well and the gel then left to set for 30 minutes. Once set, the gel was clamped in place, and both top and bottom tanks were filled with TBE buffer. The loading well was flushed out, and the denatured DNA samples (90°C for 3 minutes) were loaded. The gel was then left to run at a constant power setting of 65 Watts for 3 hours. Once run, the gel was transferred to a pre-cut sheet of 3MM Whatman paper, covered with Saran Wrap and dried on a vacuum gel dryer. When dry, the gels were exposed to X-ray film for between 2-24 hours at room temperature then processed.

2.7.2 Fluorescent dye sequencing

A more convenient approach to sequencing was to label plasmid or PCR-generated DNA with fluorescent dNTPs and sequence it automatically using an Applied Biosystems automated sequencing machine 373A. The method was performed according to the manufacturer's instructions. Typically, 100ng PCR-generated double-stranded DNA or 1µg of double-stranded DNA was labelled using an ABI Dyedeoxyterminator cycle sequencing kit (ABI 401113). This was carried out in a PCR machine. Since only a single primer is used, DNA templates are linearly amplified. Each of the dideoxynucleotides was labelled with a different fluorescent dye and so the four chain termination reactions were carried out in the same tube and electrophoresed simultaneously. Once run, the data was analysed using Applied Biosystems 373A software.

2.8 Protein manipulations

2.8.1 SDS-PAGE analysis of proteins

Discontinuous SDS-polyacrylamide gels (Laemmli, 1970) were used for the separation of proteins under denaturing conditions. Gels were poured using the Mini-Protean II dual slab cell for miniature polyacrylamide gels (BioRad, 165-2940). The

gel comprised a resolving (lower) gel and a stacking (upper) gel, the latter of which acts to concentrate large sample volumes, resulting in better resolution.

The gel apparatus was set up and filled with resolving gel monomer solution¹. This was immediately overlaid with H₂O and allowed to polymerise. Once this had been achieved, the H₂O was removed and the stacking gel monomer solution² was immediately poured on top, the comb was fitted and the acrylamide was left to polymerise. Once polymerised, the gel apparatus was fitted into the buffer tank which was filled with Tris-glycine running buffer³.

Protein samples which had been denatured by boiling in 2x SDS loading buffer⁴, were loaded onto the gel along with 3µl pre-stained molecular weight 'Rainbow markers' (Amersham RPN796). The gel was run at 150-200 volts. The gel was removed from the glass plates and stained with Coomassie brilliant blue dye (Bio-Rad, 161-0400)⁵ for 10 minutes. Excess stain was discarded and the gel was destained overnight in methanol destain solution⁶. The destained gel was dried onto a piece of 3MM Whatman paper at 80°C under vacuum.

¹ Resolving gel monomer solutions

	7.5%	10%	12%
distilled water	4.85ml	4.05ml	3.35ml
1.5M Tris.Cl pH 8.8	2.5ml	2.5ml	2.5ml
10% SDS	100µl	100µl	100µl
30/0.8% acrylamide	2.5ml	3.3ml	4.0ml
10% APS	50µl	50µl	50µl
TEMED	5µl	5µl	5µl
Total	10ml	10ml	10ml

² Stacking gel monomer solution

	<u>4%</u>
Distilled H ₂ O	6.1ml
0.5M Tris.Cl pH6.8	2.5ml

10% SDS	100μl
30%/0.8% acrylamide	1.3ml
10% APS	50μl
TEMED	10μl
Total	10ml

³ Tris glycine buffer, pH 8.3

25mM Tris
250mM glycine
0.1% SDS

⁴ SDS-loading buffer (2X)	<u>stock</u>	<u>50ml</u>
62.5mM Tris.Cl pH 6.8	0.5M	6.25ml
2% (w/v) SDS	10%	10ml
2% (v/v) β-mercaptoethanol		1ml
10% glycerol	50%	10ml
0.1% bromophenol blue	1%	5ml
H ₂ O		17.75ml

⁵ Coomassie brilliant blue dye solution

0.1% (w/v) Coomassie blue dye
50% methanol
10% acetic acid

⁶ Methanol destain solution

5% methanol
7% acetic acid

2.8.2 Western blot analysis

Western blotting was carried out using the Millipore graphite electroblotter system-type II (MBBDGE002).

2.8.2.1 Transfer

Samples to be blotted were separated by SDS-PAGE and the gel was equilibrated in cathode buffer¹. The gel was laid on a piece of nitro-cellulose (Schleicher and Schuell, BA83 0.2µm, 401396) which had been cut to the same size as the gel and soaked in cathode buffer. This was laid on top of three sheets of Whatman 3MM paper, the bottom of which had been soaked in anode buffer No.1² and the other two which had been soaked in anode buffer No.2³. On top of this was placed 3 sheets of 3MM paper which had been cut to the same size as the gel and also soaked in cathode buffer. Air bubbles were removed from the 'sandwich' by rolling with a glass pipette. Any trapped air bubbles would affect transfer of the proteins. A current of 2.5mA/cm² of filter paper was passed through the apparatus for 40 minutes. Transfer was shown to be complete by observing that the molecular weight markers had transferred to the membrane.

¹ cathode buffer	0.025M Tris
pH9.4	0.04M glycine
	20% methanol

² anode buffer No.1	0.3M Tris
pH10.4	20% methanol

³ anode buffer No.2	0.024M Tris
pH10.4	20% methanol

2.8.2.2 Blotting

The filter from 2.8.2.1 was blocked by immersion in blocking solution¹ for 1hr at room temperature. The primary antibody was added at the appropriate dilution in blocking solution containing 0.02% sodium azide, and the filter was incubated overnight with agitation at room temperature. The next day the 1° antibody was removed and stored, and the filter was washed three times in TBST² for 10 minutes each time. The horse-radish peroxidase-conjugated (HRP) 2° antibody was added at a 1/5000-

1/10000 dilution in TBST and the filter was incubated for 1 hour at room temperature. The filter was again washed three times for 10 minutes each time with TBST. The filter was developed using the ECL system (Amersham, RPN190) according to manufacturer's instructions.

¹ Blocking Solution	Dried milk	5%
	Tris.Cl pH 7.5	25mM
	NaCl	50mM
	(NaN ₃	0.02%)
² TBST	NaCl	150mM
	Tris pH 8.0	20mM
	Tween-20	0.05%

2.8.3 *In vitro* translation

This was carried out using the Promega TNT rabbit reticulocyte lysate system. The pET6H vector used in these assays carried a bacteriophage T7 promoter. ³⁵S-methionine 10μCi/μl (Amersham) was used as the label. During the labelling reaction, the manufacturer's instructions were followed. In order to test incorporation of label, 5μl of a 50μl labelling reaction was run on a 12% SDS-PAGE gel.

2.9 *E.coli* manipulations

2.9.1 Strains

The following strains were used for plasmid and library propagation throughout this work :

- XL1-Blue:** F' :: Tn10 *proA*⁺*B*⁺ *lacI*^q Δ(*lacZ*)*MI5/ recA1 endA1 gyrA96 thi hsdR17* (r_K⁻m_K⁺) *supE44ΔU169 relA1 lac*
- JA226:** *recBC leuB6 trpE5 hsdR*⁻*M*⁺ *lacY600*

- DH5α:** F-*endA1 hsdR17*(r_k⁻, m_k⁺) *supE44 thi-1 λ⁻ recA1 gyrA96 relA1 Δ(argF-lacZYA)U169* (ϕ80d *lacZ ΔM15*)
- BNN132:** *endA1 gyr96 hsdR17 relA1 supE44 thiΔ(lac-proAB)*
[F' *traD36 proAB⁺ lacI^q Z ΔM15*] λKC (*kan-cre*)
- JM109:** *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)* F' [*traD36 proAB⁺ lacI^Q lacZΔM15*]
- BL21 DE3:** pLysS: F' *ompT hsdS_b*(r_b⁻ m_b⁻)

2.9.2 Media and growth conditions

Luria-Bertani Broth (LB): This was used for routine bacterial growth

Bacto tryptone	10g
NaCl	10g
Yeast extract	5g
H ₂ O	to 1 litre

For solid media, 15g/litre Bacto agar (Difco, 0140-01) was added. Following electrotransformation of bacterial cells (section 2.9.4.1), the cells were resuspended in 1ml of LB containing 10mM MgCl₂.

Terrific Broth: Used for large scale preparation of plasmid DNA

Bacto-tryptone	12g
Bacto yeast extract	24g
Glycerol	4ml
H ₂ O	to 1 litre

After autoclaving 100ml of salt solution was added.

KH ₂ PO ₄	2.31g
K ₂ HPO ₄	12.54g
H ₂ O	to 100ml

Selective antibiotics

Antibiotic stock solutions were stored at -20°C and were added to autoclaved media.

Ampicillin

A x1000 stock solution of 50mgml⁻¹ was dissolved in H₂O.

Tetracycline

A x500 stock of 5mgml⁻¹ was dissolved in ethanol.

Growth conditions

All bacteria were grown at 37°C in an orbital shaker.

Storage conditions

All bacteria were stored on LB plates for up to 4 weeks at 4°C. Long term storage was in LB medium containing 25%(v/v) glycerol frozen at -70°C. Bacteria were revived from long term storage by removing a small scraping with a disposable inoculating loop, streaking onto selective LB media and incubating at 37°C.

2.9.3 Plasmid DNA extraction and preparation

2.9.3.1 Small scale protocols

Three approaches to small scale plasmid preparation were used. All of these yielded between 5-10µg of DNA. TELT mini-preps and 'Quick' mini-preps provided DNA that could be digested with restriction enzymes but which was not suitable for use as a template for sequencing. In this case, small scale CsCl mini-preps were used. The use of kits such as 'Wizard Minipreps' (Promega) and 'Qiagen spin plasmid' kit (Qiagen, 27104) provided very clean DNA which was suitable for cloning, sequencing and transformation.

2.9.3.1.1 TELT mini-preps

A 5ml overnight culture grown in terrific broth under antibiotic selection, was spun down, the supernatant removed and the pellet resuspended in 0.8ml TELT buffer. To this was added 40µl of 100mgml⁻¹ lysozyme. The tube was left for 2 minutes at room temperature, boiled for 2 minutes and cooled on ice for 10 minutes. Cell debris was spun out for 10 minutes at 4°C. The supernatant was removed to a fresh tube, 480µl of isopropanol was added and the tube was left on ice for 2 minutes. DNA was spun out at 14K for 20 minutes at 4°C. The pellet was washed in 70% ethanol, air dried and resuspended in 50µl H₂O.

TELT buffer

50mM	Tris.Cl pH7.5
62.5mM	EDTA pH8.0
2.5M	LiCl
0.4%	Triton X-100

2.9.3.1.2 'Quick' mini-preps

5ml of bacteria grown overnight at 37°C in LB or terrific broth was spun down and resuspended in 200µl of Solution I. Immediately, 200µl of Solution II was added and the tube was inverted several times until the mixture had cleared. At this point 200µl of Solution III was added and the tube was inverted several times. The tube was spun for 10 minutes at room temperature following which the supernatant was removed to a fresh tube and precipitated by the addition of 1/10th volume of 3M NaOAc (pH 7.4) and 2 volumes of ice-cold absolute ethanol. After cooling the tube at -70°C for 15 minutes it was spun for 15 minutes at 4°C. The pellet was washed in 70% ethanol, air dried and resuspended in 50µl of H₂O.

Solution I: 50mM glucose
25mM Tris.Cl pH 8.0
10mM EDTA pH 8.0

Solution II: 0.2M NaOH

1% SDS

Solution III: 3M KOAc

2M acetic acid

2.9.3.1.3 Small scale CsCl mini-preps

These were based on the method of Saunders and Burke, (1990) and essentially, the DNA was made in the same way as for the 'Quick' mini-preps (2.9.3.1.2) except that following removal of the supernatant (380µl), it was precipitated by the addition of 720µl of isopropanol and centrifuged for 5 minutes at room temperature. Once the pellet was washed and dried it was redissolved in 100µl TE. 100mg CsCl was added and dissolved then 10µl of ethidium bromide (10mgml⁻¹) was added. The tube was spun for 2 minutes to pellet out cell debris and the supernatant was carefully removed to a fresh tube whereupon the ethidium was removed by extracting three times with 50µl of isopropanol. Following this, 400µl TE was added to the cleared solution. The DNA was precipitated by the adding 50µl of NaOAc and 720µl of isopropanol and centrifuging for 5 minutes at room temperature. The pellet was washed in 70% ethanol, air dried and resuspended in 20µl of TE/ H₂O.

2.9.3.1.4 Small scale plasmid preparation using commercially available kits

The two kits used were the Wizard Mini-prep kit (Promega) and the Qiagen Spin Plasmid kit (Qiagen, 27104). DNA prepared by these kits was done so according to the manufacturer's instructions.

2.9.3.2 Large scale plasmid preparation

In order to make large amounts of plasmid DNA the methods described earlier were not suitable and consequently had to be scaled up. One of these methods is a scale-up of the alkaline lysis method (Birnboim and Doly, 1978) followed by CsCl gradient centrifugation. This method of plasmid preparation is more suitable for the prepara-

tion of DNA libraries due to its high yield and purity. The second method involved the use of commercially available kits

2.9.3.2.1 Caesium chloride plasmid preparation

A 400ml culture of *E.coli* cells carrying the plasmid of interest was grown overnight at 37°C in terrific broth containing the appropriate antibiotic. The cells were placed on ice for 30 minutes then spun down in 200ml bottles in a Sorvall GSA rotor at 8000rpm for 10 minutes. The cell pellets were pooled and thoroughly resuspended in 20ml of Solution I containing 10mgml⁻¹ lysozyme. 40ml of Solution II was added, mixed and the solution left on ice for 10 minutes. 30ml of cold solution III was added, the suspension mixed well and left on ice for 30 minutes. The cell debris was then spun out at 8000rpm for 10 minutes. The supernatant was filtered through muslin into a clean centrifuge bottle. The DNA and any residual protein was precipitated by the addition of 0.6 volumes of isopropanol. After mixing thoroughly, the DNA was pelleted by centrifuging as before. The pellet was washed in 70% ethanol and air-dried. Prior to ultracentrifugation in a Beckmann T-100 rotor, the DNA pellet was resuspended in 2.2ml TE along with 2.4g CsCl and 200µl of ethidium bromide (10mgml⁻¹). This mixture was injected into heat sealable tubes to just below the neck of the tube. The tube was balanced to within 5mg against a blank or against another sample which was to be centrifuged. The tubes were sealed and spun overnight at 80000rpm at 16°C. After centrifugation the plasmid band was removed using a sterile needle and syringe and placed in an eppendorf tube. The ethidium was extracted by addition of an equal volume of water-saturated butan-1-ol. This step was repeated until all traces of ethidium had been removed. Once done, the DNA was precipitated by adding 2.5 volumes of 75% ethanol and centrifuging for 15 minutes at room temperature. The pellet was washed with 70% ethanol, air-dried and resuspended in 2ml TE. The DNA was then re-precipitated by adding 0.1 volumes of NaOAc, 2 volumes of ethanol and centrifuging as before. The pellet was washed in 70% ethanol, dried and resuspended in an appropriate volume of H₂O (100µl-500µl). The DNA concentration was measured using a spectrophotometer.

2.9.3.2.2 Large scale plasmid preparation using commercially available kits

The 2 kits used in this instance were the 'Wizard Maxiprep' kit (Promega) and the 'Qiagen Maxiprep' kit (Qiagen, 28104). Although quicker than the previous method, the yield of DNA obtained by this method was variable.

2.9.4 Bacterial transformation

2.9.4.1 Competent cell preparation

1 litre of LB was inoculated with 1/10th of the volume of a fresh overnight culture of the cells of interest. This was grown at 37°C with vigorous shaking until the culture had reached an OD₆₀₀ of 0.5-1.0. At this point the flask was chilled on ice for 30 minutes then centrifuged in a Sorvall GS3 rotor for 10 minutes at 8000rpm. The cells were washed with an equal volume of ice-cold H₂O, centrifuged as before, washed with 1/2 the volume of H₂O and re-centrifuged. The cell pellets were pooled, resuspended in 20ml of 10% glycerol and centrifuged as before. The pellet was then resuspended in 3ml of 10% glycerol such that the cell concentration was about 3x10¹⁰ cells/ml. This suspension was aliquoted into cryotubes (200µl/tube), frozen in liquid N₂ and stored at -70°C until required. Cells prepared in this manner can be stored for up to 6 months.

2.9.4.2 Electrotransformation

50µl of the cells to be transformed were placed in an eppendorf tube on ice. 1-2µl of the DNA to be transformed was mixed with the cells and the suspension was pipetted into a cold 0.2cm electroporation cuvette (Bio-Rad) on ice. The Gene Pulser apparatus (Bio-Rad) was set to 25µF, 200Ω and 2.5kV and the cuvette containing the DNA and cells was placed in the chamber. Following pulsing, 1ml of pre-warmed LB-Mg₂⁺ was added and the cuvette incubated at 37°C for 1hr. The cells were then plated onto selective media, and incubated overnight at 37°C.

2.10 Fission yeast manipulations

2.10.1 Fission yeast strains

These were all derived from the strains 972 h⁺^L and 975 h⁻^S (Leupold, 1958)

2.10.2. Media and growth conditions

Complete media

Fission yeast strains were routinely grown in or on complete Yeast Extract (YE / YPD, Difco 0127-01-7) media at 25°C. This temperature is suitable for growth of both temperature-sensitive (*ts*) strains. Cold-sensitive (*cs*) strains were grown at 30°C. YE contains the following ingredients per litre:-

yeast extract	5g
glucose	20g
(Difco-Bacto agar	20g)
Difco peptone	5g
adenine	75mg
H ₂ O	to 1 litre

Minimal media

In order to select for nutritional prototrophs, EMM (Moreno *et al.*, 1991) was used as a minimal medium. This is a modification of EMM2 (Nurse, 1975) and contains the following per litre:-

glucose	20g
(Difco-Bacto agar	20g)
NH ₄ Cl	5g
KH phthalate	3g
Na ₂ HPO ₄	1.8g
(KOH 1M	1ml)
salts	20ml

vitamins	1ml
minerals	100μl

Salts (per litre):- NaSO_4 , 5g; CaCl_2 , 750mg; MgCl_2 , 50g
 vitamins (per litre):- inositol, 10g; nicotinic acid, 10g; calcium pantothenate, 1g; biotin, 10mg.
 minerals (per litre):- H_3BO_3 , 5g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 5.2g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2g; H_2MoO_4 , 1.44g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 400mg; Citric acid, 10g and KI, 100mg.

The salts, vitamins and minerals were made up in distilled H_2O , autoclaved and stored at 4°C .

Additional growth supplements such as adenine, leucine and uracil were added to a final concentration of $75\mu\text{gml}^{-1}$ after autoclaving. Thiamine was made up as a 100mM stock solution in H_2O , filter-sterilised and stored in the dark at 4°C . It was used at a final concentration of $2\text{--}4\mu\text{M}$.

Malt extract (ME) media

In order to induce sporulation and meiosis in diploid cultures, malt extract media was used. Liquid media contained 30gl^{-1} malt extract (Difco), supplemented as necessary, whereas the solid media also contained 20gl^{-1} agar.

Phloxin B

In order to visualise dead or diploid cells on solid media, the vital stain phloxin B was added to a final concentration of 10mgml^{-1} . Phloxin accumulates in dead cells which become dark red in colour. Since colonies of diploid cells contain more dead cells than haploid colonies, it can be used to distinguish haploid colonies from diploid colonies.

MBC

The microtubule destabilising drug methyl benzimidazol-2-yl carbamate (MBC) was used to check the drug-resistance of different alleles of *mts2*. The drug was dissolved in H_2O , filter sterilised and added to complete media at the required concentration after autoclaving.

Strain isolation

Strains were isolated from frozen stocks by scraping the surface of the frozen stock with a plastic loop and streaking the cells onto a plate containing complete or selective media. The plate was then incubated at the appropriate temperature until large colonies had formed.

Growth conditions

Following the isolation of fission yeast strains from frozen stocks they were grown at 25°C or 30°C, depending on their genotype, on the appropriate solid YE media. The same temperatures were used for growth in liquid media.

Storage

Fission yeast strains can be kept for up to 2 weeks in liquid media or for up to 4 weeks on solid media, without phloxin B, at 4°C. Following this, viability decreases rapidly and consequently the strains must be stored at -70°C in media containing 15% (v/v) glycerol.

2.10.3 Genetic analysis

2.10.3.1 Crossing strains

Strains were crossed by mixing together freshly isolated cells of opposite mating types (h^+ and h^-) on a ME plate. A loopful of sterile H₂O is then used to thoroughly mix the cells. The plate was incubated at 25°C for two days to allow formation of zygotic asci. Sporulation was confirmed both by microscopic examination and by exposure of the crosses to iodine vapour which reacts with the starch in the ascus wall of sporulating diploid cells, and turns blue black. The progeny of the crosses were examined either by tetrad analysis or by random spore analysis.

2.10.3.2 Tetrad analysis

A loopful of a two day old cross was streaked in a line onto a YE plate. The asci of crossed strains were pulled using a micromanipulator (Singer instruments, UK) and

the plate was incubated for 4 hrs at 35°C or overnight at 20°C to allow the walls of the asci to break down. Each ascus was then dissected to liberate the 4 spores which were then placed in line 6mm apart using the micromanipulator. This was performed for 18-20 asci. The spores were incubated at 25°C until they had grown, then patched for further analysis.

2.10.3.3 Random spore analysis

A loopful of a three day old mating mix was resuspended in 1ml sterile distilled H₂O containing 5µl of β-glucuronidase (Glusulase) and incubated at room temperature for 12-16hrs. The mix was examined to check for complete breakdown of the asci walls. The spore concentration was counted using a haemocytometer and then 200-1000 spores/plate were plated on YE or selective medium.

2.10.3.4 Diploid construction

This was based on the use of the two complementing *ade6* alleles: *ade6-M210* and *ade6-M216*. A heterozygous diploid carrying both alleles is prototrophic for adenine whereas haploids carrying either of these alleles are adenine auxotrophs. In addition, haploids and the allele which they carry can be distinguished by the red/pink colour they turn when plated on media lacking adenine. The strains of interest which carry the adenine alleles were crossed in the normal way, incubated overnight at 25°C to facilitate conjugation then streaked onto media without adenine. Colonies were tested for their ability to sporulate.

2.10.3.5 Stability test

Following transformation of a diploid strain with a piece of linear DNA carrying a gene disrupted with a nutritional marker, usually uracil, this test was used to check for the integration as opposed to episomal maintenance of the exogenous DNA. If integration has occurred, the acquired phenotype will not be lost whereas if the DNA is replicating autonomously, the phenotype will be lost in the absence of selection. Transformants carrying the nutritional marker were replica-plated onto selective media to reduce background growth. Once grown, they were replica-plated onto YE four successive times with an overnight incubation of 25°C between each plating. These

transformants were replica-plated to selective media, grown and then streaked to single colonies on YE. The colonies were replica-plated to selective media. Transformants from which all colonies grew were considered to be stable.

2.10.3.6 Generation of h^+/h^{90} diploid strains

Following stable integration of the disrupted *let1* gene into the *S.pombe* genome, the isolation of sporulating diploids was prevented by a rearrangement at the mating type locus. Thus, instead of being heterozygous h^+/h^- at that locus, the cassettes at the mating type locus had recombined to become either h^+/h^+ or h^-/h^- homozygotes. The non-sporulating diploids were mated with haploids of known mating-type to assess which rearrangement had taken place. Those diploids which had rearranged to become h^+/h^+ at the mating type locus were used to generate h^+/h^{90} revertants which would then sporulate. Reversion was done by plating 10^5 cells on non-selective media, growing for 3 days at 25°C, then replica plating onto ME media to look for sporulating diploids. In this way, sporulating diploids carrying a disruption of the *let1* gene were isolated.

2.10.3.7 Screen for *cs* revertants of *mts2*

The *mts2* mutant was streaked to single colonies on YE plates and incubated at 25°C for 3 days. From these plates, a total of 100 colonies was picked into separate eppendorf tubes containing 1ml of YE liquid media and incubated for 2-3 days at 25°C. This resulted in a saturated 1ml culture containing approximately 10^9 cells. From each tube 0.3ml (3×10^8 cells) was plated onto each of 100 YE plates, containing phloxin B, which were then incubated at 36°C. After 4 days of incubation, between 2-10 colonies had grown on each plate. Two colonies were picked from each plate and tested for growth at 18°C on complete media containing phloxin B

2.10.4 Lithium acetate transformation of *S.pombe*

The lithium acetate procedure (Moreno *et al.*, 1991) resulted in a transformation frequency of 10^3 - 3×10^3 transformants/ μ g of pRS305 DNA. 100ml of cells were grown overnight at 25°C to an OD₆₀₀ 0.5-1.0 (10^7 - 2×10^7 cells/ml) in YE or EMM with supplements. The cells were harvested, washed in 20ml H₂O then in 20ml 0.1M LiOAc

pH 4.9, then resuspended in 1ml of 0.1M LiOAc. To 100µl of cells in an eppendorf tube was added 0.5µg-1.0µg of DNA, 50µg denatured salmon sperm DNA or tRNA and 370µl of filter-sterilised 50% PEG3350 (Sigma, P-3640) which had been pre-warmed at 25°C. The cells were mixed well and incubated at 25°C for 1hr. The cells were then heat-shocked at 46°C for 30 minutes, spun down, resuspended in 1ml YE and incubated at 25°C for 1-3 hrs. Following this, 300µl of the mix was plated onto selective media and incubated at 25°C until colonies had formed.

2.10.5 Preparation of nucleic acids from fission yeast

2.10.5.1 Recovery of plasmid DNA

This is based on the method of Winston (1987). Plasmids can be extracted from overnight cultures or from patches on solid media. Either a 1ml overnight culture is centrifuged and resuspended in 200µl of extraction buffer¹, or a loopful of cells from a 2-3 day old patch is resuspended in the same buffer. To this suspension was added 0.2ml of phenol-chloroform-isoamyl alcohol (25:24:1) and 0.2ml of acid washed glass beads (0.5cm, BDH). The cell suspension was vortexed for 2 minutes then spun for 5 minutes in a microcentrifuge. The supernatant was removed and ethanol precipitated and the DNA pellet was resuspended in 20µl of H₂O. 5µl was then used to transform either JA226, the use of which prevents plasmid rearrangement due to the *recBC* genotype, or XL1-Blue. Transformants were selected on media containing ampicillin.

¹Plasmid extraction buffer

triton X-100	2%
SDS	1%
NaCl	100mM
Tris.Cl pH 8.0	10mM
EDTA	1mM

2.10.5.2 Preparation of genomic DNA

10ml of a stationary phase culture was harvested in a 15ml Falcon tube (5 minutes at 2500rpm). The supernatant was discarded, the cells were resuspended in 1.5ml CPS¹ containing 2.5mgml⁻¹ Zymolyase 100T (ICN Biomedicals, 320931) and incubated for 1hr at 37°C. The cells were then harvested (2 minutes at 2500rpm and resuspended in 300µl of 5xTE. 35µl of 10% SDS(w/v) was added and the mixture was incubated at 65°C for 5 minutes. Following addition of 100µl of 5M potassium acetate (pH 5.6) the mixture was incubated on ice for 30 minutes. Cell debris was pelleted by centrifuging at high speed for 15 minutes at 4°C. The supernatant (~400µl) was removed and added to 1ml of ice cold ethanol. This was centrifuged for 10 minutes at 4°C. The pellet was washed in 70% ethanol, resuspended in 400µl of 5xTE containing 100 µg ml⁻¹ RNase (Sigma, R-5000) and incubated at 37°C for 2-4 hours. Proteins were removed by extracting once each with an equal volume of phenol, phenol-chloroform and chloroform. The remaining aqueous phase was removed to another tube, precipitated and washed with 70% ethanol. The pellets were air-dried and dissolved in 100µl of TE. A 10ml culture yielded 50-100µg of genomic DNA.

¹CPS

50mM citrate-phosphate buffer pH 5.6

1.2M sorbitol

0.1% (v/v) β-mercaptoethanol

2.10.6 Preparation of protein extracts from fission yeast

This method produces total non-denatured fission yeast protein extracts which were used for western blots. It was also used to make protein extracts from *S. cerevisiae*. 200ml cultures in mid-exponential phase (OD₆₀₀ 0.5 - 1.0) were harvested in four tubes (2500rpm for 5 minutes). The supernatants were discarded and each cell pellet was resuspended in 100µl of cell lysis buffer¹ and transferred to an eppendorf tube. Acid washed glass beads (0.5mm, Sigma G-9268) were added to just below the meniscus of the liquid. Cells were broken by vigorous vortexing for 30 seconds followed by 30 seconds on ice, over a 30 minute period. Cells were pelleted by centrifugation at high speed for 5 minutes at 4°C. The supernatant was removed to another

eppendorf tube and the insoluble debris pelleted by centrifugation for 15 minutes at 4°C. Supernatants were transferred to a clean eppendorf tube and stored at -70°C. Protein concentrations were assayed using the Bio-Rad assay kit (500-0002). Prior to loading samples on an SDS/PAGE gel, an equal volume of 2xSDS-sample buffer was added and the proteins boiled for 2 minutes then cooled rapidly on ice.

¹ Lysis buffer	<u>stock</u>	
50mM KCl	3M	17µl
50mM Tris.Cl pH 7.9	1M	50µl
25% glycerol	50%	500µl
2mM DTT (Sigma, D-0632)	1M	2µl
0.1% triton X-100	1%	100µl
Chymostatin (Sigma,C7268)	5mgml ⁻¹	5µl
Antipain (Sigma, A-6271)	5mgml ⁻¹	5µl
Leupeptin (Sigma, L-2884)	5mgml ⁻¹	5µl
Pepstatin (Sigma, P-4265)	5mgml ⁻¹	5µl
PMSF (Sigma, P-7626) ^a	100mM	2µl

^aPMSF was stored at 4°C and added to the buffer just before use. All other protease inhibitors were stored at -20°C, as was DTT.

2.11 Budding yeast methods

During the course of this work, *S. cerevisiae* was used extensively for protein interaction screening using the yeast 2-hybrid system. Although some methods, such as the extraction of protein, are identical to those used with fission yeast, others differ greatly.

2.11.1 Budding yeast strains

Y187 MATα *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, -112 met*
URA3::GAL---lacZ

Y190 MATa *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, -112 + URA3::*
GAL---lacZ, LYS2::GAL(UAS)---HIS3 cyh^r

2.11.2 Media and growth conditions

Synthetic complete (SC) media

The strains shown above were routinely grown on solid SC media, containing additional nutrients, at 30°C. SC media contains the following ingredients per litre:

glucose	20g
Bacto-agar	20g
Yeast nitrogen base	6.7g
supplements	0.4g (Bio 101 inc.)
adenine	40mg
(histidine	40mg)

In order to select for protein interactions, 3-aminotriazole (3-AT, Sigma A-8056) was included at concentrations of 25mM or 50mM. In this case, histidine was not added to the media.

2.11.3 Transformation

100ml of cells to be transformed were grown overnight in YEPD media to an OD₆₀₀ of 0.5-1.0. The cells were harvested at 5K for 10 minutes in a Sorvall centrifuge, washed with 40ml of distilled H₂O and resuspended in 20ml of LiAcTE¹. The cells were pelleted and resuspended in 1ml LiAcTE. This step was repeated twice. To 50µl of cells was added 50µg of salmon sperm DNA, 0.5-1.0µg of plasmid DNA and 300µl of 40% PEG 3350 in LiAcTE. The cells were mixed thoroughly and incubated at 30°C for 30 minutes followed by an incubation at 42°C for 30 minutes. Cells were allowed to recover from the heat shock by being resuspended in 1ml of SC-Leu, Trp

for 1-3 hrs at 30°C. 50 µl of cells were plated onto solid selective media and incubated at 30°C for 3-5 days.

¹ LiAcTE	100mM lithium acetate
	10mM Tris pH 8.0
	1mM EDTA

2.11.4 X-GAL filter lift assay

Colonies which grew on selective media were tested for the interaction of plasmid-encoded proteins by assaying the β-galactosidase activity on solid media. Whatman 3MM paper was cut to the same size as a petri dish then overlaid onto the patches which had been grown for 2 days at 30°C. Once the filter paper was saturated it was removed from the plate and immediately immersed in liquid nitrogen for 10 seconds in order to permeabilise the cells. Once thawed it was laid cells upwards in a petri dish on top of a second 3MM disc which had been soaked with 0.3ml/square inch of Z-buffer¹ containing 1mg/ml X-Gal. The filters were incubated at 30°C to allow colour to develop.

¹Z-Buffer per litre (pH. 7.0)

Na ₂ HPO ₄	16.1g
NaH ₂ PO ₄	5.5g
KCl	0.75g
MgSO ₄ .7H ₂ O	0.25g
2-mercaptoethanol	2.7ml

2.11.5 β-galactosidase liquid assay

10ml of an exponentially growing culture (OD₆₀₀ 0.5-1.0) was spun down and resuspended in 0.3ml of cell lysis buffer¹ and transferred to an eppendorf tube. The tube was immersed in liquid nitrogen for 5 minutes then thawed slowly. This freeze-thawing step was repeated 3 times to ensure maximum cell lysis. Once lysis was complete, 0.2ml of the cells were transferred to a fresh eppendorf tube. To these cells was added 0.8ml of Z-buffer, 0.2ml of ONPG (4mg/ml) and 80µl of X-Gal

(20mg/ml). The suspension was mixed and incubated at 30°C to allow the cleavage of the ONPG to take place. This was visualised as the appearance of a yellow colour and was quantified by measurement of the OD₄₂₀. Calculation of the β-galactosidase activity as a measure of the interaction of 2 proteins in the 2-hybrid system was made by application of the following formula:

$$\frac{1000 \times \text{OD}_{420}}{V \times T \times [\text{protein}]}$$

V = volume of cells (0.2ml)
T = time taken for yellow colour to develop

2.11.6 Mating of *S.cerevisiae*

Patched colonies to be mated were replica-plated onto selective media and allowed to grow overnight. At the same time, a lawn of a yeast strain of the opposite mating type MAT α was also grown up. The following morning, the plate carrying the patched colonies containing the library plasmid of interest and of mating type MAT α were mixed with a loopful of the strain of the opposite mating type carrying the control plasmids and the resulting mixture of diploid MAT α /MAT α cells were patched onto solid YEPD plates and incubated overnight at 30°C. In the morning these patches were replica-plated onto SC -Leu, -Trp and allowed to grow. Colonies which formed were patched onto SC -Leu, -Trp and SC -His, +3AT and subsequently tested for their β-galactosidase activity.

2.12 Cytological methods

2.12.1 Staining of cells

2.12.1.1 Propidium Iodide (PI) staining

Staining with propidium iodide was used prior to analysing the DNA content of cells by FACS analysis. Since PI binds to DNA and RNA, the RNA has to be removed prior to analysing the cells. 5ml (or approximately 2×10^8 cells) of an exponentially

growing culture was centrifuged and the pellet resuspended in 1ml of ice-cold 70% ethanol. This suspension can be kept at 4°C until required and is left for at least 12 hours. The cells were pelleted, the supernatant aspirated and the cells washed in 1ml 50mM sodium citrate pH 7.0. The cells were pelleted again, resuspended in 1ml of 50mM sodium citrate containing 25µl 10mg/ml RNase A (Boehringer) and incubated at 37°C for 2 hrs. A small sample of cells was taken and stained by addition of an equal volume of 2.5µg/ml PI (Sigma, P-2804) in sodium citrate. Complete degradation of RNA was confirmed by visualisation of PI staining using a Zeiss fluorescence microscope prior to analysing the DNA content.

2.12.1.2 Staining of septal material with aniline blue

This is based on the method of Kippert *et al.* (1995) and is a useful way in which to stain the septa of both live and fixed *S.pombe* cells. Cells to be stained, whether fixed in ethanol or paraformaldehyde, were washed once with PBS and mounted in dye solution (0.5mg/ml in PBS) for 10 minutes at room temperature. Staining was visualised under a Zeiss Axioplan fluorescence microscope.

2.12.1.3 DAPI staining of *S.pombe* cells

4', 6'-diamidino-2-phenylindole (DAPI) is used as a fluorochrome to stain the nuclei of live and fixed cells. Cells to be stained were dried onto a glass slide then a drop of DAPI, diluted in Vectashield to a final concentration of 0.2µg/µl, was placed on top.

2.12.1.4 Paraformaldehyde fixation and anti-tubulin staining of cells

1. Dissolve 15.2g paraformaldehyde in 40ml PEMS¹ + 1ml of 5M NaOH at 70°C to make a 38% (w/v) solution.
2. Cells to be stained are grown in YEPD (haploid cells, 25°C) or EMM+ supplements (spores at a concentration of 3x10⁶/ml, 20°C) overnight.
3. At the first time point 10ml of cells are removed from the culture into a 50ml falcon tube. To this is added 10ml of YEPD/EMM + 2.4M sorbitol and 2.2 ml

of 38% paraformaldehyde.

4. Incubate sample at 36°C for 30 minutes.
5. Spin down cells and wash in 5ml of PEMS.
6. Spin down cells and resuspend in 1ml PEMS.
7. Spin down cells and resuspend in 1ml PEMS + 0.5mg/ml zymolyase T100.
8. Incubate at 36°C for 90 minutes.
9. Spin cells and wash in 1ml PEMS.
10. Resuspend cells in 1ml PEMS + 1% triton X-100. Leave at room temperature for 5 minutes.
11. Wash cells in 1ml PEM². Repeat 3 times.
12. Resuspend cells in 0.5ml PEMBAL³.
13. Spin cells and resuspend in 0.25ml of PEMBAL. Incubate at room temperature for 30 minutes.
14. Remove 50 µl of cells to a fresh eppendorf. Spin for 10 seconds, rotate tube 180°C and repeat.
15. Remove supernatant and add 100µl of a 1/15 dilution of TAT1 α-tubulin antibody in PEMBAL.
16. Rotate overnight at room temperature.
17. Wash cells x3 in PEMBAL.
18. Add 100µl of CY3-anti mouse IgG (diluted 1/100 with PEMBAL). Incubate overnight at room temperature.
19. Wash cells x 3 in 150µl of PEMBAL
20. Wash cells in 150µl of PBS. Resuspend cells in this and store until required.

¹PEMS

PEM
1M sorbitol

²PEM 100mM PIPES pH 6.9
 1mM EGTA
 1mM MgSO₄

³PEMBAL PEM

1% (w/v) BSA
0.1% (w/v) sodium azide
0.1M lysine

2.13 Analysis of stained cells

2.13.1 FACS analysis of PI stained cells

The DNA content of cells stained with PI was measured using a Becton Dickinson Fluorescence Activated Cell Sorter (FACS). Stained cells were sonicated for 5 seconds to obviate any clumping which may have occurred, then analysed using Cellfit and Lysys software. Data was stored on floppy disk. Since the only parameter being measured was that of the PI staining, which fluoresces in the FL2 channel, all other detectors were switched off to minimise file size. The FL2 detector was regularly set at an amplitude of 900 and the amplifier at a level of 1.5. A cell flow rate of 300-700 was regarded as optimal.

2.13.2 Image capture and analysis of stained cells

Stained cells, on poly-lysine coated slides, were screened with a Zeiss Axioplan fluorescence microscope equipped with a triple band-pass filter set (Chroma). This allowed sequential visualisation of FITC, Texas Red and DAPI images using a computer-driven excitation filter wheel. As the polychroic filter and emission filter remained in place while acquiring the images, image registration was perfect. Metaphase arrested cells were imaged using a cooled CCD camera fitted with a KAF400 chip (Photometrics). Separate images of nuclear staining and tubulin staining were pseudocoloured using an Apple Macintosh Quadra 900 computer. These images were stored on portable hard discs (SyQuest) and were printed using a Colour Ease dye sublimation printer.

Chapter 3

Chapter 3 Isolation of genes whose products interact with Mts2p

3.1 Introduction

Analysis of the original *mts* mutants has provided a wealth of information regarding subunit composition and subunit interactions of the 26S proteasome in fission yeast. This is because in many cases, the mutations have been shown to lie in genes encoding subunits of the 26S complex (Gordon *et al.*, 1993, 1996 and unpublished results: Wilkinson *et al.*, 1997). In at least two cases, the crossing of two independent *mts* mutants, *mts1-1* with *mts3-1*, or *mts2-1* with *mts4-1*, results in an inviable double mutant at the permissive temperature (Wilkinson *et al.*, 1997, C.Gordon, pers. comm.). This 'synthetic lethality' (Guarente, 1994) indicates a potential physical interaction between the mutant gene products.

As a means of finding genes whose products interacted with the product of the *mts2⁺* gene (termed Mts2p), a number of strategies were used. These encompassed both classical and molecular genetics. Two of these approaches, pseudoreversion analysis and suppressor screening, were unsuccessful for reasons which will be discussed. However, a more recently developed strategy, which proved successful, was also used. This method, the 'yeast 2-hybrid system', provides a means of selecting for strong or weak physical interactions between two proteins.

3.2 Isolation of spontaneous *mts2 ts⁺* revertants by pseudoreversion

Pseudo-reversion analysis was originally used as a means to find spontaneous extragenic suppressors, of conditionally lethal mutants, which themselves would have a conditional phenotype (Jarvik and Botstein, 1975). This type of screen was also used

to look for *cs* suppressors of a *ts* mutation in the *S.cerevisiae act1-1* mutant (Adams and Botstein, 1989; Novick *et al.*, 1989).

Since the *mts2-1* mutant was *ts*, this technique could potentially be used to identify suppressors of the *ts* phenotype. When plated at a low restrictive temperature of 18°C, it might be expected that cells carrying a *cs* suppressor of *mts2-1* would either be inviable or would grow poorly. The rate of spontaneous mutation in *S.pombe* is approximately 1×10^{-7} (Moreno and Nurse, 1991). Therefore, by plating a large number of cells, spontaneous mutations could be screened for their ability to suppress the *ts* defect of *mts2-1*.

A screen was performed, in order to identify *cs* revertants of *mts2-1* (described in Materials and Methods). Of 400 colonies which grew at the high temperature (36°C), none showed any growth defect at the lower temperature (18°C), indicating that none of the suppressor mutations had a *cs* phenotype. Although this method could be used to find suppressors of the *ts* phenotype of *mts2-1*, these suppressor mutations did not have a *cs* phenotype. They could have been either intragenic revertants of the original *mts2-1* mutation, or novel extragenic suppressors.

The failure to isolate any *cs* suppressors of *mts2-1* suggests that the generation of spontaneous *cs* mutations in other proteins that might interact with Mts2p is an event which occurs at a very low frequency. In the original experiments to detect suppressors of the *act1-1* mutant (Novick *et al.*, 1989), 5% of the revertants were *cs*. This may have been due to the structural role performed by the actin protein. Alternatively, as mentioned earlier, a mutation in a gene encoding an interacting subunit may, in conjunction with *mts2-1*, result in a dominant synthetically enhanced lethality (Guarente, 1994). The inviable nature of the resulting mutant would preclude the isolation of any potential suppressors by this classical method.

3.3 Isolation of suppressors of *mts2-1* by screening cDNA and genomic DNA libraries

The production of yeast genomic libraries has been facilitated by the use of shuttle vectors such as pWH5 or pDB248 which, due to the presence of both bacterial and yeast origins of replication, are able to be propagated in both species (Beach *et al.*, 1982). These libraries represent a powerful molecular genetic tool for the isolation of genomic DNA fragments which, when transformed into the yeast mutant strain of interest, can suppress the deleterious phenotype caused by the mutation in question. One problem with this system is that the analysis of the cloned DNA by sequencing is often slow due to the large sizes of the DNA insert and the presence of introns. Also, the expression of any genes within the genomic fragment is not regulated. Therefore, although suppression of mutations may require a high level of expression of the cloned DNA fragment, for other mutations, the same overexpression may be deleterious.

The production of yeast cDNA libraries has obviated many of the problems associated with the use of genomic DNA libraries. These are made by reverse transcribing the messenger RNA (mRNA) and directionally cloning it into a shuttle vector under the control of the strong thiamine repressible *nmt* (no message in thiamine) promoter (Maundrell, 1990; Maundrell *et al.*, 1993). The directionally cloned cDNA can then be expressed at different levels by varying the concentration of thiamine in the media. A concentration of 4 μ M thiamine is sufficient to repress almost all expression from the *nmt* promoter.

An *S.pombe* cDNA library (a gift from Chris Norbury) in the vector pREP3X (pMBS36LEU) had been used to clone the *mts1*⁺ (unpublished result), *mts2*⁺ (Gordon *et al.*, 1993), *mts3*⁺ (Gordon *et al.*, 1996), *mts4*⁺ (Wilkinson *et al.*, 1997, in press) and *pad1*⁺ (Penney *et al.*, 1997, unpublished) gene. By the use of a *Mus musculus* cDNA library, cloned into the same vector, an extragenic multi copy suppressor of *mts2-1* had been isolated. When analysed, this was shown to be MSS1, the murine homologue of an ATPase subunit (S7) of the 26S proteasome (Gordon *et al.*, 1993). This result suggested that screening the *S.pombe* cDNA library for multi copy suppressors of *mts2-1* might also yield the *S.pombe* homologue of MSS1.

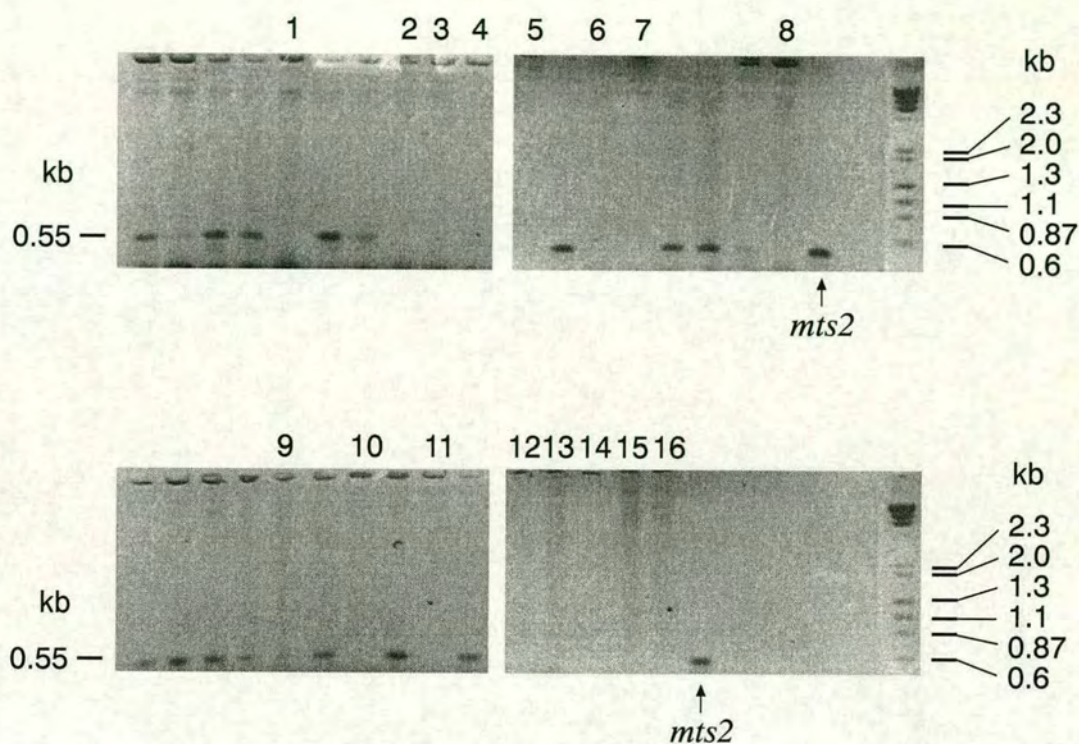


Figure 3.1 PCR of cDNA suppressors of the *mts2-1* mutant.

Plasmid DNA, from 32 yeast colonies which grew at 35°C on media lacking leucine, was transformed into the bacterial strain *XL-1 Blue*. Colonies were then used in a PCR reaction using oligonucleotides C823 and D858, which were directed against the *mts2⁺* gene. The PCR products were run on a 0.8% TAE gel. Lanes 1-16 were negative for the diagnostic 0.55kb PCR product. All other lanes, including the *mts2⁺* cDNA positive control (shown), were positive for this product

Approximately 6×10^5 transformants were screened for their ability to rescue the *ts* defect and leucine auxotrophy of *mts2-1* at 36°C. Thirty two colonies which grew at 36°C were selected for analysis. Having tested the plasmid stability of these transformants, the plasmid DNA was re-transformed into bacteria and subjected to the following analyses:

3.3.1. PCR Analysis

The plasmids, which had been transformed into bacteria, were screened by PCR to identify those that contained a copy of the *mts2⁺* gene. This PCR reaction was performed using oligonucleotides C823 and D858 which were complementary to sequences within the *mts2⁺* gene. Figure 3.1 shows the result of a PCR reaction carried out on DNA from plasmids which rescued the *ts* phenotype of *mts2-1*. The result shows that the diagnostic PCR product, from the *mts2⁺* gene, is present in 16 of the 32 cDNA suppressors shown (unmarked lanes).

3.3.2 Southern blot analysis

Of the 32 cDNAs analysed, 16, that by PCR appeared not to contain the *mts2⁺* gene (Figure 3.1, lanes 1-16), were digested to completion with *SalI* and *BamHI* to release the cDNA insert. Figure 3.2 (A) shows that 7 of the plasmids (lanes 3, 4, 6, 7, 9, 10 and 15) contain an insert of a size very similar to that of the *mts2⁺* cDNA. Mts2p belongs to a family of ATPases, the AAA family (Confalonieri and Duguet, 1995), some of which have very similar molecular weights to Mts2p. It is possible that these 6 plasmids might carry DNA inserts which encode other members of this AAA family. However, when the digested DNA was transferred to a nylon membrane and then hybridised to a ^{32}P -labelled probe complementary to a non-conserved region of the *mts2⁺* gene, all of the inserts that were the same size as *mts2⁺* gave a strong hybridisation signal (Figure 3.2 (B) lanes 3, 4, 6, 7, 9, 10 and 15). In addition, 1 more (lane 1) gave a weak signal of the same size as *mts2⁺*, indicating that this probably also contained the *mts2⁺* cDNA insert. The inserts in lanes 2, 5, 8, 13, 14 and 16, which were of a different size to the *mts2⁺* cDNA, failed to hybridise to the *mts2* probe,

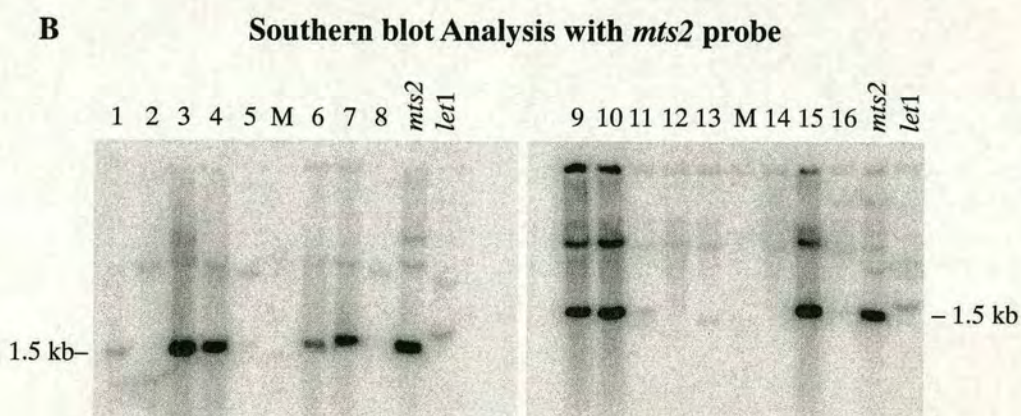
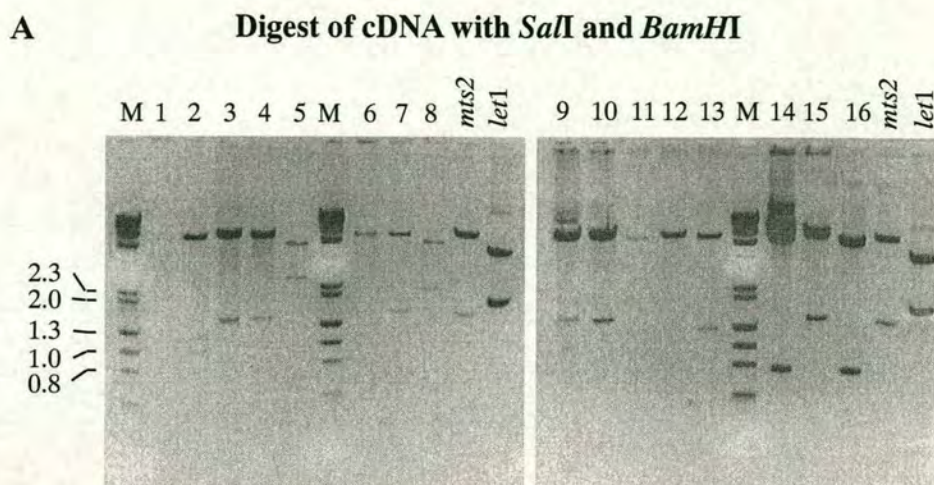


Figure 3.2 Restriction digest and Southern blot of plasmid DNA from cDNA suppressors of *mts2-1*

Plasmid DNA from cDNA suppressors of *mts2-1*, which were negative for the PCR reaction shown in Figure 3.1, was digested to completion with *Sall* and *Bam*HI and run on a 0.8% TAE gel (A). In addition, *mts2*⁺ cDNA in pREP1 and *let1*⁺ genomic DNA in pFL20 were also digested and used as controls. The gel from (A) was Southern blotted using a ³²P-labelled PCR product from the *mts2*⁺ gene (B). The filters were washed twice in 0.1 x SSC / 0.1% SDS at 68°C. Lanes 1,3,4,6,7,9,10 and 15 give the same size of band (1.5kb) as the *mts2* control. Lanes 2,5,8,14,16 have an insert of a different size from that of the *mts2* control. Position of DNA markers (M) are shown.

indicating that they were unlikely to be related to *mts2*⁺. No inserts were visible in lanes 11 and 12.

3.3.3 Retransformation of potential suppressor cDNAs into *mts2-1*

All of the cDNAs analysed by Southern blotting were used to retransform *mts2-1* to leucine prototrophy. Once colonies had grown they were replica-plated onto selective media and incubated at 36°C. All of the colonies that grew at this restrictive temperature contained the cDNAs that gave a positive signal when probed with labelled *mts2*. This result confirmed that the only suppressor plasmids which were able to rescue the *mts2-1* mutation were those that carried the *mts2*⁺ cDNA. The results of these experiments are summarised in Table 3.1

3.3.4 Summary of approach

In using this approach to look for cDNA suppressors of *mts2-1*, 32 cDNAs were analysed. Of these, the only cDNAs that were able to rescue the *ts* defect of *mts2-1* at 35°C were those which encoded the *mts2*⁺ gene. Sequencing of these potential suppressors confirmed that they contained the *mts2*⁺ gene (data not shown). On one occasion, *mts2*⁺ was isolated as a fusion to a cDNA encoding the *S.pombe* cyclophilin gene (de Martin and Philipson, 1990). When sub-cloned, the gene encoding cyclophilin did not rescue the *mts2-1* mutant (data not shown). On 3 occasions it was found as a fusion to cDNAs encoding different *S.pombe* ribosomal genes. Again, the ribosomal genes alone could not rescue the *mts2-1* mutant. Although ribosomal genes occur naturally as fusions to ubiquitin, at least in *S.cerevisiae* (Bartel *et al.*, 1989), this phenomenon has not been observed for other genes. Consequently, it is likely that the fusions to *mts2*⁺ have occurred as artefacts during the construction of the cDNA library.

The results shown above raise 2 questions: Firstly, why did this type of screen not facilitate isolation of the *S.pombe* homologue of MSS1 as a cDNA that suppressed the *ts* defect of *mts2-1*, given that MSS1 had been isolated in the same way? It may

be that there is no MSS1 homologue present in *S.pombe*. This is clearly unlikely since *CIM3*, the budding yeast homologue of this gene, has been identified (Ghislain *et al.*, 1993; Schnall *et al.*, 1994), and also because the murine MSS1 gene rescues the *ts* phenotype of the *mts2-1* mutant (Gordon *et al.*, 1993). A more likely reason is that the *S.pombe* homologue of MSS1 is not present in this library. This may have occurred during initial library construction or during subsequent amplification. This

Table3.1 Results of *mts2* cDNA suppressor analysis

Suppressor	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	<i>mts2-1</i> +
Insert Size kb	?	1.1	1.5	1.5	?	1.5	1.5	?	1.5	1.5	0.9	0.8	1.3	0.6	1.5	0.6	1.5
Southern blot (+ve / -ve)	+	-	+	+	-	+	+	-	+	+	-	-	-	-	+	-	+
Re-rescue of <i>mts2-1</i> (+/-)	+	-	+	+	-	+	+	-	+	+	-	-	-	-	+	-	+

Key to table ? - Unknown insert size

idea is supported by the results of Southern blotting of library DNA with a labelled MSS1 probe This experiment failed to produce any hybridisation signal even under conditions of low stringency, suggesting that the *S.pombe* MSS1 homologue is not represented in this library (data not shown).

Secondly, does this mean that there are no other genes that, when over-expressed, will rescue *mts2-1*? The failure to find any suppressors of *mts2-1* may be due to the stringent conditions under which the screens have been performed. That is, the stabilisation of a heat labile protein by the overexpression of an interacting protein may be very inefficient at such a restrictive temperature.

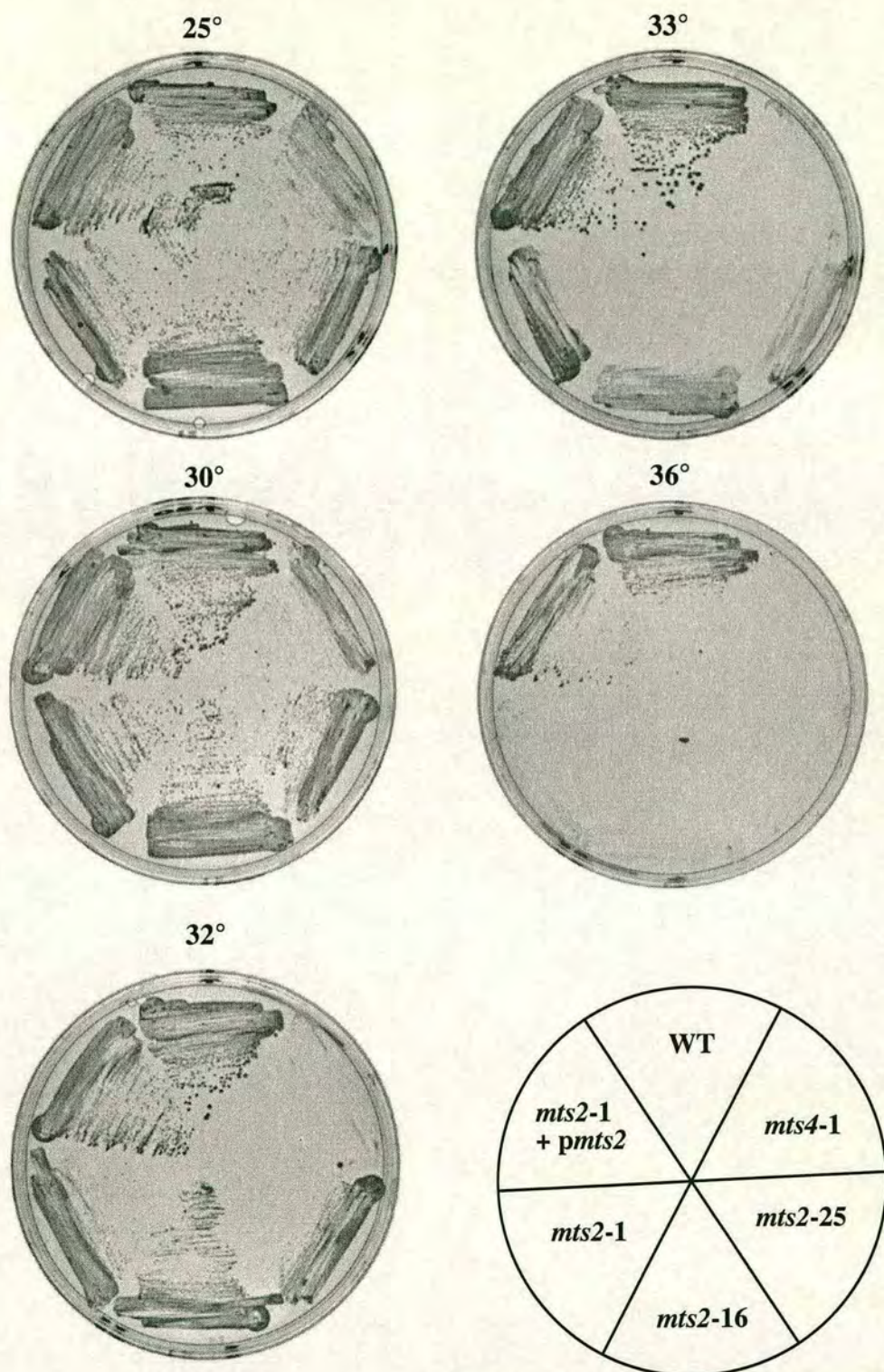


Figure 3.3 Temperature sensitivity of mutant alleles *mts2-1*, *mts2-16* and *mts2-25*

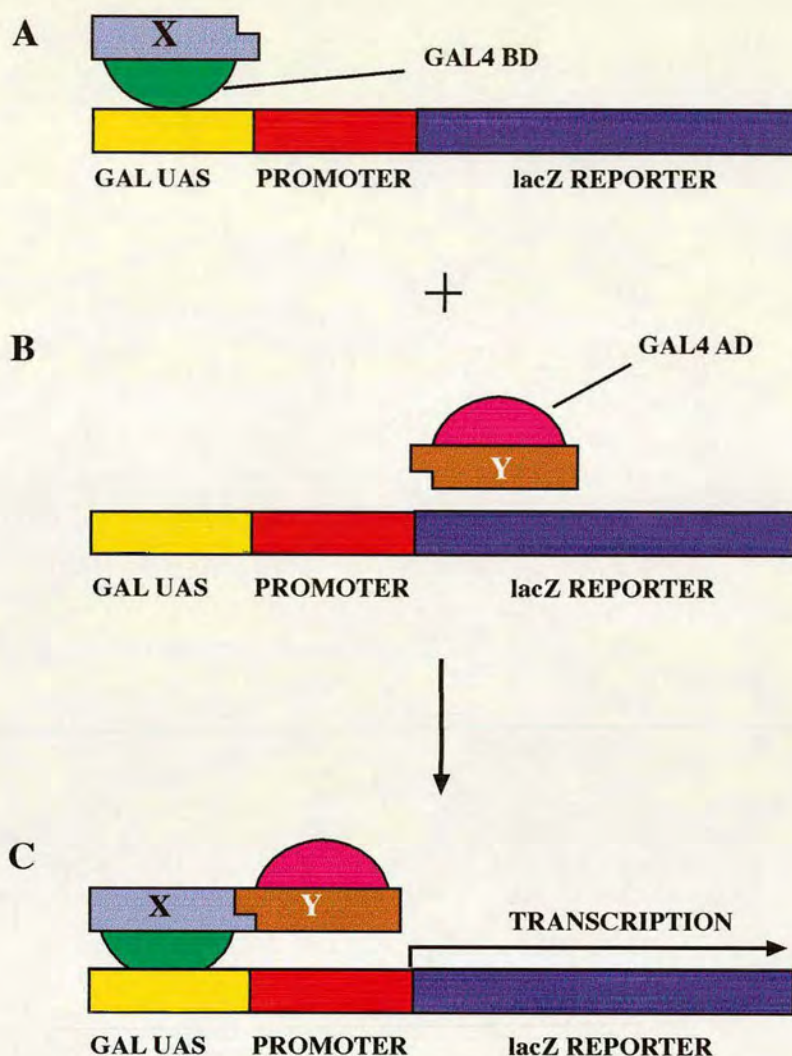
mts2-1, *mts2-16* and *mts2-25* strains were streaked onto complete media and incubated at 25°C, 30°C, 32°C, 33°C and 36°C until colonies had appeared. As controls, *mts2-1* carrying the *mts2*+cDNA, and *mts4-1* were used.

When a similar screen was used to look for suppressors of the *mts4-1* mutant, at a lower temperature of 32°C, a number of extragenic cDNA suppressors were isolated. One of these was found to be *mts2*⁺ (Wilkinson *et al.*, 1997 and pers. comm.). Even at 32°C, *mts4-1* fails to grow. Since *mts2-1* grows well at this temperature (Figure 3.3), this screen could be repeated at lower temperatures such as 34°C or at 33°C. Alternatively, other alleles of *mts2* such as *mts2-25* (see Figure 3.3 and Chapter 6) could be used. This allele is more *ts* than the *mts2-1* mutant.

3.4 Isolation of interacting proteins by use of the yeast 2-hybrid system.

The yeast 2-hybrid system (Fields and Song, 1989; Chien *et al.*, 1991) was developed as a means for identifying *in vivo* interactions between two proteins. It is based on the functional reconstitution of the *S.cerevisiae* *GAL4* transcriptional activator from two protein domains, a *GAL4* DNA binding domain and a *GAL4* activation domain which are encoded on different plasmids, and the consequent transcriptional activation of a nutritional reporter gene (*HIS3*), and a *lacZ* reporter gene, under the control of a *GAL4*-responsive promoter. The DNA-binding domain is fused to a 'bait' protein, and the activation domain is fused to a library-encoded 'target' protein. This system is shown schematically in Figure 3.4. Non-specific activation of the *HIS3* reporter by either plasmid can be obviated by inclusion in the growth medium of the histidine anti-metabolite 3-aminotriazole (3-AT).

The *S.pombe* 2-hybrid library used in these experiments was obtained from D. Beach. It contained *S.pombe* cDNAs cloned into the *EcoRI-XhoI* site and fused to the *GAL4* activation domain in the 'prey' plasmid pGADGH (see Figure 2.5), which carries the *LEU2* nutritional marker. The 'bait' plasmid contained the *mts2*⁺ cDNA fused to the *GAL4* DNA-binding domain in the plasmid pAS1-CYH2 (Figure 2.3) to give the construct pAS-*mts2*⁺. As well as carrying the *TRP1* gene as a selectable nutritional marker, pAS1-CYH2 also carries a copy of the *CYH2* gene. Cells carrying this gene are sensitive to the protein synthesis inhibitor, cycloheximide. The *S.cerevisiae* host strain used in these experiments, *Y190*, carries a recessive mutation in the *CYH2* gene



GAL4 BD = Gal4 BINDING DOMAIN $\alpha\alpha$ 1-147

GAL4 AD = Gal4 ACTIVATION DOMAIN $\alpha\alpha$ 768-881

GAL UAS = UPSTREAM ACTIVATION SEQUENCE

UNDER CONTROL OF Gal4 PROTEIN

X = TARGET PROTEIN

Y = CANDIDATE PROTEIN

Figure 3.4 Schematic diagram of yeast 2-hybrid system.

A host strain is co-transformed with the vector pAS-CYH2 carrying the gene for a 'bait' or 'target' protein (X) fused to the *S.cerevisiae* GAL4 DNA-binding domain (A), and the vector pACT1 / pGADGH which carries a gene encoding a 'prey' protein (Y) fused to the *S.cerevisiae* GAL4 activation domain (B). The interaction between 'bait' and 'prey' results in the reconstitution of a functional Gal4 transcriptional activator and the concomitant expression of the *lacZ* gene which is under the control of the GAL4 promoter (C).

which confers resistance to cycloheximide. These properties were used for both the mating experiment, and for removal and analysis of the library plasmid.

Throughout these experiments, the interaction between the products of *SNF1* in pAS1 and *SNF4* in pACTII (Figure 2.4) was used as a positive control for detecting and measuring β -galactosidase expression. *SNF1* (Sucrose Non-Fermentation) and *SNF4* encode two subunits of a protein kinase involved in the expression of glucose-repressible genes in response to glucose deprivation in *S.cerevisiae* (Celenza *et al.*, 1989). In addition, pAS1-*SNF1* was used as a negative control to test the specificity of interaction of constructs in plasmids expressing the *GAL4* activation domain. In the same way, pACT1-*SNF4* was used as a negative control for non-specific activation by constructs in plasmids expressing the *GAL4* DNA-binding domain.

3.4.1 Test for non-specific interaction of pAS-*mts2* with pACT-*SNF4*

pAS-*mts2*⁺ was transformed into the *S.cerevisiae* strain Y190 (*MATa leu2-3 his3 trp1*) and stable *TRP* prototrophs were selected. These colonies were used to detect any interaction between Mts2p and SNF4. Competent Y190 cells carrying pAS-*mts2*⁺ were transformed with pACT-SNF4. No interaction was observed between these two proteins. This was judged by the inability of colonies to grow on media containing 25mM 3-AT. In addition, colonies which grew on selective AH media had no obvious β -galactosidase activity. pAS-*mts2*⁺ was therefore used as the 'bait' protein in this 2-hybrid screen.

3.4.2 Screening an *S.pombe* 2-hybrid library using Mts2p as a bait protein

The *S.pombe* library was transformed into competent Y190 cells that were already carrying pAS-*mts2*⁺. The colonies which grew varied in size. Only the large colonies were picked because it seemed that these were more likely to reflect a strong interaction between Mts2p and other library plasmid-encoded proteins. From this initial transformation, 108 colonies were picked and tested for their ability to express β -galactosidase. Of these, 86 that had been grown on media containing 3-AT expressed β -galactosidase. The results of this screen are shown in Figure 3.5.

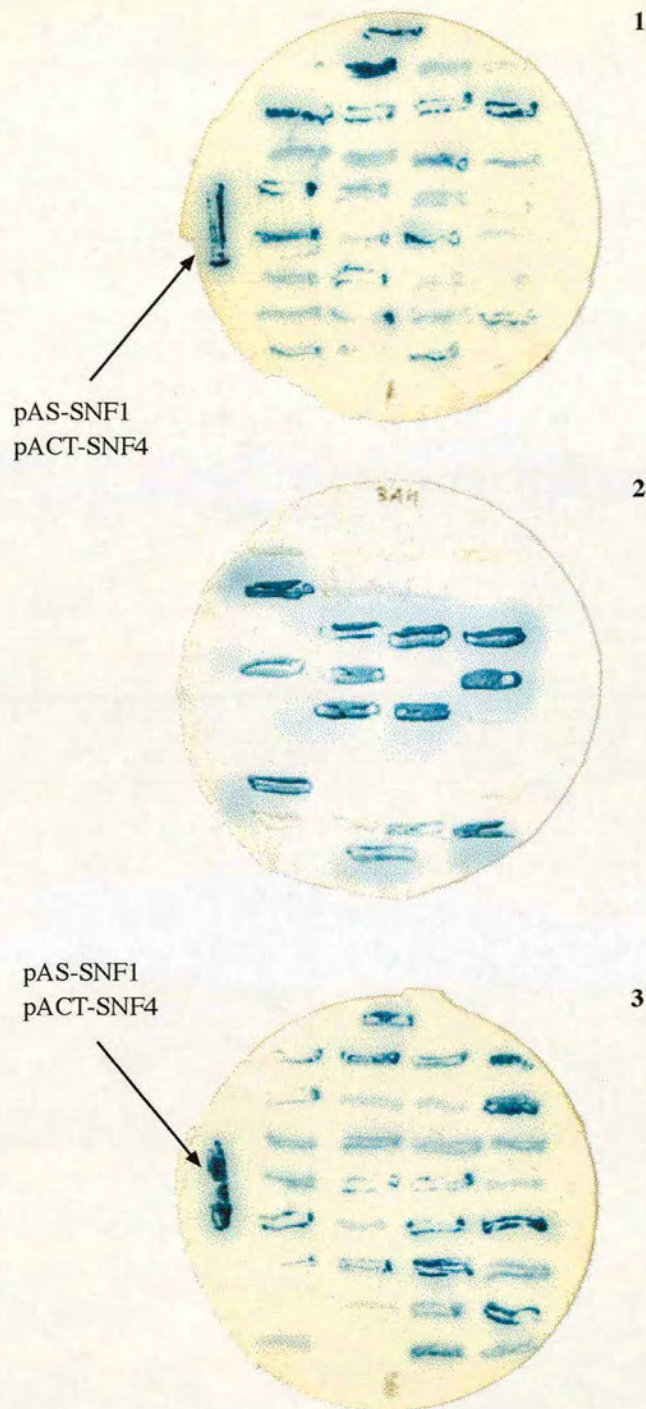


Figure 3.5 2-Hybrid library secondary screen to look for proteins which interact with Mts2p

108 colonies picked from the primary screen were patched onto 3 plates containing SC-A+3AT media and incubated at 30°C. The patches were then lysed and tested for their ability to express β -galactosidase (blue). Results are shown relative to the pAS-SNF1-pACT-SNF4 positive control.

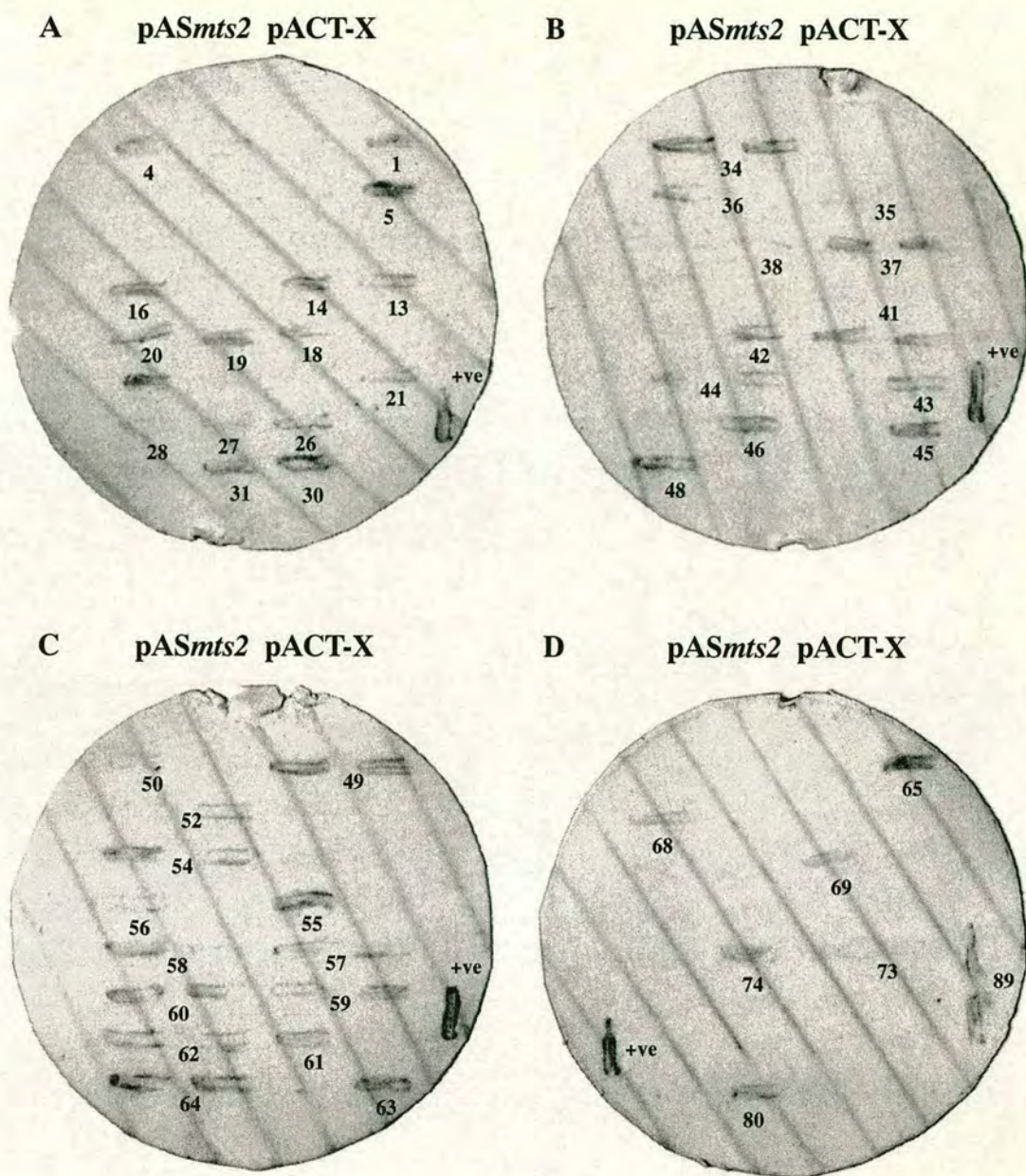


Figure 3.6 Removal of 2-hybrid false positives by mating

S.cerevisiae Y190 cells carrying library plasmids were mated to Y187 cells of the opposite mating type carrying pAS-*mts2*. Matings were patched onto media containing 3-AT, incubated at 30°C until grown, and tested for β -galactosidase activity. Numbers correspond to the number of the primary positive colony. On plate A, one colony from isolate nos. 1-32 was patched. On plate B to D, two colonies from isolate 33 - 90 were patched. Patches which expressed β -galactosidase activity are numbered. 50 of the original 108 isolates still expressed β -galactosidase.

3.4.3 Mating test for non-specific activation of β -galactosidase expression

The mating test for non-specific activation of β -galactosidase expression involved mating two yeast strains of opposite mating type. One of these, Y190 (*MATa*) would contain only the plasmids isolated from the library screen, thereby necessitating removal of the bait plasmid pAS-*mts2*⁺. The other strain Y187 (*MAT α*) would contain pAS-X, X being a gene whose product was unrelated to *mts2*⁺. The mating test was performed as described (see Materials and Methods). Of the 86 mating diploids, none expressed β -galactosidase when mated to Y187-pAS-*SNF1*, the negative control. However, when mated to Y187-pAS-*mts2*⁺, 50 still expressed β -galactosidase (Figure 3.6).

3.4.4 Analysis of genes encoding interacting proteins

Prior to the mating experiment, library plasmid DNA from 10 of the 86 secondary isolates was analysed. Restriction analysis of the plasmid DNA, using the enzymes *EcoRI* and *XhoI*, indicated that there were four different sizes of insert. One member of each class was sequenced using oligonucleotides F444 and F250, which are complementary to the multiple cloning site (MCS) of the plasmid pGADGH and lie on either side of the cDNA insert. The sequences obtained were used to search the Genbank and EMBL databases using UWGCG software.

The DNA sequence from the first class of isolate (2H1,4 and 5) was identical to that of a previously identified gene from *S.pombe* called *let1*⁺. This gene was originally identified as an essential gene at the mating-type locus (Michael *et al.*, 1994). In addition, a mutation in the *S.cerevisiae* homologue of *let1*⁺, called *SUG1*, was identified as a suppressor of a deletion in the acidic activation domain of the *GAL4* transcriptional activator (Swaffield *et al.*, 1992). The inserts from two further classes of isolates (plasmids 2 and 8) displayed homology to DNA encoding yeast ribosomal proteins (rpl15 and rpl29). The final class of isolate bore no strong homology to any

ctgcaggaaatcggcaccgagaaatgacggaggtgctgaagactaacgttttacagagtaa 38
 EcoRI M T E V L K T N V L Q S N 13
 tgagaacatagttcagttattatccccaaaaattcaagatgctgaacttgctatttctcca 98
 E N I V Q Y Y T Q K I Q D A E L A I L Q 33
 aaagacacaaaatttacgacgattagagggtcaaagaaacggactaaatgcaagagttcg 158
 K T Q N L R R L E A Q R N G L N A R V R 53
 ctttctaagagaggagattcagttgcttcaggaacccggaagttagtgcggtgaagtgat 218
 L L R E E I Q L L Q E P G S Y V G E V I 73
 aagacaatgggaaagaacaaagttctagtcaaagtcocatcctgaaggcaaatatgtcgt 278
 K T M G K N K V L V K V H P E G K Y V V 93
 tgatattagtcagatattgatattaaggagattaaacctaatattcgtgtagccttgag 338
 D I S P D I D I K E I K P N I R V A L R 113
 gaacgattcataccaattaattaaaatcctcccaacaagggttgatccacttgctctctct 398
 N D S Y Q L I K I L P N K V D P L V S L 133
 catgatggtcgaaaaaattcctgactccacttatgagatgggttggtggtcttgaaaagca 458
 M M V E K I P D S T Y E M V G G L E K Q 153
 aatcaaagaaattaaagaagttatcgaattaccagtggaagcatccagaacttttgaaag 518
 I K E I K E V I E L P V K H P E L F E S 173
 ctttaggtattcctcagcctaaaggtatttcttttatgggtccccgggaacgggtaaaaac 578
 L G I P Q P K G I L L Y G P P G T G K T 193
 cttgttagotagagcagttgccaccataccgattgcaaattcatcgtgtcagtggtatc 638
 L L A R A V A H H T D C K F I R V S G S 213
 agaacttggtcaaaaagtatatattggcgaaggtagtcgtatgggtcgtgaactatttggtat 698
 E L V Q K Y I G E G S R M V R E L F V M 233
 ggctcgtgagcatgctccttctattattttcatggatgagatcgactccatcggtctcttc 758
 A R E H A P S I I F M D E I D S I G S S 253
 acgatctgattctagtggtggaagtggcgatagcgaagttcagaggaccatgttagagct 818
 R S D S S G G S G D S E V Q R T M L E L 273
 gttgaatcagcttgatggatttgaagcaacaaaaatatcaaggtcattatggccactaa878
 L N Q L D G F E A T K N I K V I M A T N 293
 ccgtatcgacatcttggtatcctgctcttcttctgctccaggtcgtattgatcgtaaaattga 938
 R I D I L D P A L L R P G R I D R K I E 313
 attccacotccatctgctgagggcacgtgccgaattttgagaatccattcgaggtccat 998
 F P P P S A E A R A E I L R I H S R S M 333
 gaatttaacacgtggcattgatttgaagagtatagcggagaaaatgaacggtgctagtgg 1058
 N L T R G I D L K S I A E K M N G A S G 353
 tgcogagctaaaaggtgtttgtactgaagccggtatgtttgcattgagagaaagacgtgt 1118
 A E L K G V C T E A G M F A L R E R R V 373
 tcatgttactcaagaagattttgaattagctgttgtaaaagtattaaacaagggcgatttc 1178
 H V T Q E D F E L A V A K V L N K G D S 393
 HindIII
 tggagaaatgagtttacaaaagcttttcaagtagatgattttacatatgtattaatgaat
 G E M S L Q K L F K * 403
 atgattataagtttagtcaaaaaaaaaaaaaaaaaaactcgagggggccc
 XhoI

Figure 3.7 Nucleotide and deduced peptide sequence of *S.pombe let1⁺* isolated from a 2-hybrid library.

The insert from 2H1 was sequenced in both directions by dideoxy sequencing. The sites used for cloning the insert into the vector pGADGH, *EcoRI* and *XhoI* are shown. The *HindIII* sites used for production of the disrupted allele (see Chapter 5), are also shown.

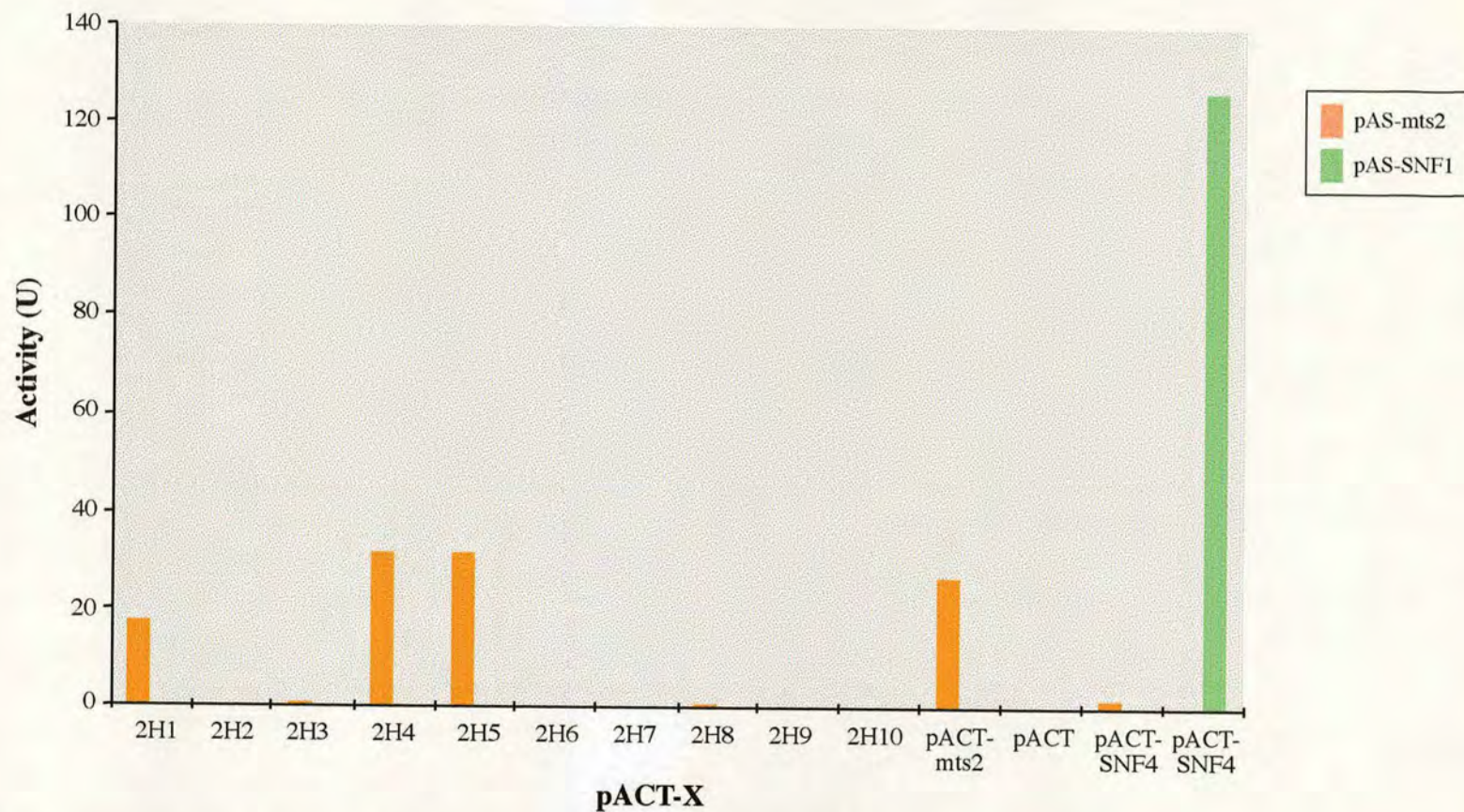


Figure 3.8 β -galactosidase activity of primary isolates 1-10 from the yeast 2-hybrid library screen.

β -galactosidase activity of each of the above isolates was measured in liquid culture. 3 patches from each isolate (1-10) were inoculated into 10ml SC+ AH liquid media and incubated at 30°C until an OD_{595} 0.2-0.6 was reached. Cells were lysed and the β -galactosidase activity, normalised to protein concentration, was measured. The activity of pAS-*mts2*⁺ with controls pACT-*mts2*⁺, pACT, and pACT-*SNF4* was also measured as was the activity of pAS-*SNF1* with pACT-*SNF4*.

entries in either the Genbank or EMBL databases. The insert which bore homology to *letI*⁺ was completely sequenced. The nucleotide and amino acid sequence of the sequenced *letI*⁺ cDNA is shown in Figure 3.7.

The plasmid DNA from these 10 isolates was co-transformed with pAS1-*mts2*⁺ back into Y190. Colonies were patched and tested for β -galactosidase activity in liquid culture. Figure 3.8 shows that only isolates 1, 4 and 5 produced any substantial β -galactosidase activity, relative to the pAS-*SNF1*/pACT-*SNF4* positive control. According to their insert size and digestion pattern, these are all members of the same class of plasmid, suggesting that they are all likely to contain the *S.pombe letI*⁺ gene. This was later confirmed by sequencing (results not shown).

Following the mating test, all 50 positive colonies were analysed by PCR to identify those which contained the *letI*⁺ cDNA. One of the oligonucleotides used in this assay, I631, was homologous to a region of the *letI*⁺ gene. The other, F444, was complementary to the MCS of the plasmid, upstream of the cDNA insert. In total (Figure 3.9 (A)), 39 of the 50 positive colonies (2H1-2H50) carried a library plasmid which contained all or a large part of the *letI*⁺ gene. Of these, one, 2H3, appeared to be slightly shorter than the other fragments suggesting an N-terminal truncation of the gene. This was subsequently verified by sequencing, and showed that the N-terminal 32 amino acids of the *letI*⁺ ORF were absent. This result will be discussed subsequently and in Chapter 4.

The remaining 11 colonies which had failed to produce a PCR product in the previous experiment were then re-amplified using the oligonucleotides F444 as before, and F250, which was complementary a region of the MCS downstream of the insert. By doing this, not only could the size of the insert be calculated, but the product could also be sequenced using the same primers. The result of this reaction is shown in Figure 3.9 (B). Of these, 6 were the same size (2H5, 2H11, 2H42, 2H48, 2H49 and 2H50), one was larger (2H10) and the other two were smaller (2H21 and 2H32). The remaining two cDNAs, 2H27 and 2H45 were the same size as each other and the same size as a PCR containing the *letI*⁺ cDNA. They were therefore assumed to be copies of the *letI*⁺ cDNA.

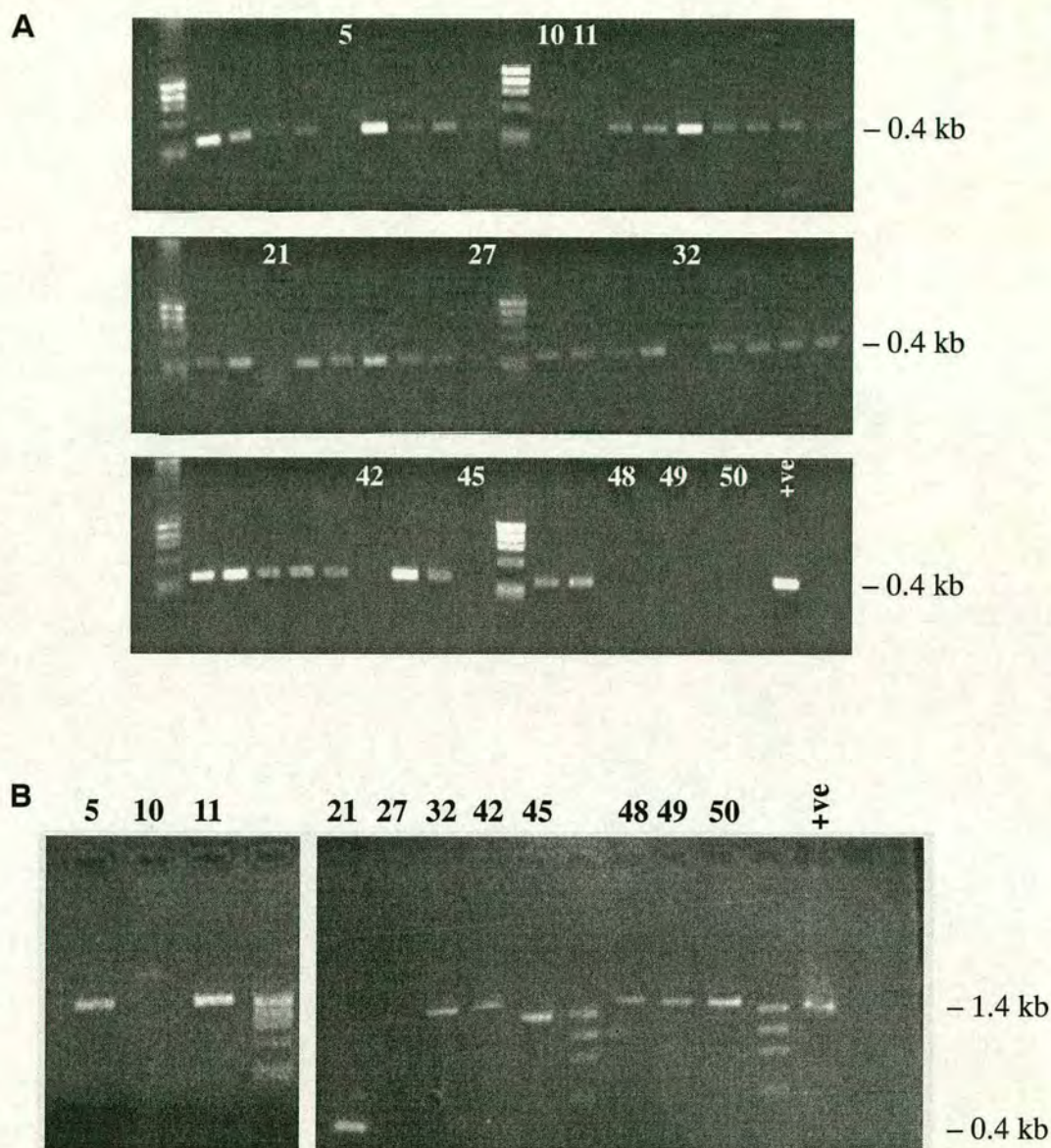


Figure 3.9 PCR screening of tertiary positives for the presence of *let1*⁺

(A) *S.cerevisiae* Y190 cells carrying library plasmids 2H1-2H50 were used as templates in a PCR reaction to look for the presence of the *let1* gene. The oligonucleotides I631, directed against the *let1* cDNA, and F444, complementary to the pGADGH multiple cloning site (MCS), were used to amplify a 0.4kb fragment of the *let1* gene. Of a 50 μ l PCR reaction, 5 μ l was run on a 0.8% TAE gel. No PCR product is visible in lanes corresponding to isolates 2H5, 10, 11, 21, 27, 32, 42, 45, 48, 49 and 50. (B) Cells that were negative in (A) were then subjected to a second PCR reaction using oligonucleotides F444 and F250 which were complementary to the pGADGH MCS and which lay on either side of the cDNA insert. The PCR products were again run on a 0.8% gel. *let1*⁺ cDNA was used as a positive control.

No sequence information could be generated from the downstream oligonucleotide F250. However, sequence information was generated from all of the PCR products using the primer F444. These sequences were analysed as before. The results of this section are shown in Table 3.2. The most striking result was that the sequence of 6 of the PCR products, which were the same size, was homologous to that of *CIM5*, the *S.cerevisiae* homologue of *S7*, an ATPase subunit of the 26S proteasome (Ghislain *et al.*, 1993). *CIM5* in turn is the homologue of the *Mus musculus* *MSS1* gene, a multicopy suppressor of the *ts* defect of the *mts2-1* mutant in *S.pombe* (Gordon *et al.*, 1993).

The sequence of 2H10 was identical to that of an *S.pombe* cosmid (accession number Z69788) sequenced in the *S.pombe* sequencing project. The putative protein encoded by this cosmid is homologous to a hypothetical yeast protein, also discovered in the sequencing project. Both of these putative proteins contain an ATP/ GTP-binding site. In addition, they both possess a stretch of lysine(K) and glutamic acid (E) residues at their N-terminus. The significance of this KEKE region will be discussed.

There was a high degree of homology between the sequence of 2H21 and the *S.pombe* homologue of the mammalian U2AF splice factor (accession number U02280, Potashkin *et al.*, 1993). However, the entire sequence encoded by the 2H21 insert was downstream of the U2AF ORF, and was in the opposite orientation. A translation of the 2H21 insert revealed a short ORF of 300 bases. The potential polypeptide encoded by this ORF bore no homology to any entries in the SWISS-PROT database. The significance and nature of this isolate is therefore unknown.

The sequence of 2H32 bore no homology to any known sequence in the database. As mentioned earlier, no sequence was obtained from the 3' end of the cDNA insert, so that the nature of this 2-hybrid isolate is unknown.

The PCR product of 2H5, whose DNA sequence resembled that of *CIM5*, was completely sequenced in both directions by 'walking' along the PCR fragment. That is, by using oligonucleotides which were designed from the sequence generated. The

ISOLATE No	LENGTH SEQUENCED (bp)	HOMOLOGY TO KNOWN GENES	OTHER FEA- TURES
2H1,2,4,6-9, 12-20, 22-31, 43-47	2H1- completely	<i>S.pombe letI</i> ⁺ gene (U02280)	
2H3	210	<i>S.pombe letI</i> ⁺ gene (U02280)	Truncation of nu- cleotides 1-88
2H5	complete (1415)	<i>S.cerevisiae CIM5</i> gene (P33299)	
2H10	421	<i>S.pombe</i> chromosome I cosmid (Z69728)	N-terminal KEKE motif.
2H11	445	<i>S.cerevisiae CIM5</i> gene (P33299)	
2H21	377	<i>S.pombe</i> splicing factor U2AF (L22577)	
2H32	210	No homology to any entries in database	
2H42	447	<i>S.cerevisiae CIM5</i> gene	
2H48	433	<i>S.cerevisiae CIM5</i> gene	
2H49	472	<i>S.cerevisiae CIM5</i> gene	
2H50	443	<i>S.cerevisiae CIM5</i> gene	

Table 3.2 Results of analysis of 2-hybrid isolates 1-50 (2H1-2H50)

complete nucleotide and amino acid sequences of this novel *S.pombe* gene isolated from the 2-hybrid library, which will be designated *aps1*⁺ (another *pombe* 26S subunit), are shown in Figure 3.10. A comparison of the peptide encoded by *aps1*⁺ to the family of S7 proteins from different species is shown in Figure 3.11. It can be seen that there is at least a 75% identity between Aps1p and all other members of this family. The putative peptide sequence is missing the extreme N-terminus including the initiator methionine codon. By comparison to the Cim5 peptide from budding yeast, it is likely that only 3 amino acid residues are missing. Efforts are currently being made to isolate the full length cDNA for suppression studies and expression of the full length Aps1 protein.

3.5 Quantification of the interaction between Mts2p, Let1p and MSS1p

The level of interaction between these proteins can be quantitated by measuring the release of o-nitrophenol from o-nitrophenol- β -D-galactoside (ONPG). The ONPG is cleaved by the β -galactosidase resulting in an accumulation of o-nitrophenol which can be measured at an OD₄₂₀. These experiments were performed prior to the isolation of the *S.pombe* homologue of *CIM5*/MSS1. Consequently, the murine MSS1 homologue was tested for the interaction of its product with that of *let1*⁺ and *mts2*⁺.

The *let1*⁺ gene was already in the vector pGADGH, an equivalent to pACTII. The *mts2*⁺ and MSS1 genes were cloned into the vector pAS1-CYH2 and pACTII and checked for any PCR generated mutations (data not shown). The constructs, including pAS1-SNF1 and pACTI-SNF4, were transformed into Y190 in pair-wise combinations. Three colonies were patched onto AH media, grown and then used in the liquid assay. The results of these interactions are shown in Figure 3.12.

The combination of pAS-SNF1/pACT-SNF4 produces a very strong interaction, whereas this is not observed between pAS-SNF1 and any of the other pACT constructs. This result indicates that there is probably no non-specific interaction

human	..MPDYVLGAD	QRKTKREDE	..	KDDKPIRALD	EGDIALLLKTY	GQSTYSRQIK	QVEDDDIQQLL	56
rat	..MPDYVLGAD	QRKTKREDE	..	KDDKPIRALD	EGDIALLLKTY	GQSTYSRQIK	QVEDDDIQQLL	56
xenopus	..MPDYVLGAD	QRKTKREDE	..	KDDKPIRALD	EGDIALLLKTY	GQSTYSRQIK	QVEDDDIQQLL	56
mouse	..MPDYVLGAD	QRKTKREDE	..	KDDKPIRALD	EGDIALLLKTY	GQSTYSRQIK	QVEDDDIQQLL	56
celegans	..MPDHLGDD	MRKTKKDDTK	..	EEENNFQALD	EGDIALVLRKY	AQSTYSRQIK	TLADDTLNCIL	58
cerevisiae	MPPKEDWEKY	KAPLEDDDKK	..	PDDDKITVPLT	EGDIQVLLKSY	GAAPVYAAKLLK	TLADDTLNCIL	60
pombe	MPPKEDWEKY	QKPVDTTEENDKNPPPLD	EGDIEELKSY	ATGPVYARELA	ATKEDTEAVL	58
human	KKINELTGIR	ESDTGLAPPA	..	LWDLAADKQQT	LQSEEQPLQVA	RCTKII	..	102
rat	KKINELTGIR	ESDTGLAPPA	..	LWDLAADKQQT	LQSEEQPLQVA	RCTKII	..	102
xenopus	KKINELTGIR	ESDTGLAPPA	..	LWDLAADKQQT	LQSEEQPLQVA	RCTKII	..	102
mouse	KKINELTGIR	ESDTGLAPPA	..	LWDLAADKQQT	LQSEEQPLQVA	RCTKII	..	102
celegans	KKVNELSGVR	ESDTGLAPPA	..	LWDLAADKQQA	LQSEEQPLQVA	RCTKII	..	104
cerevisiae	ARIKKAKAGVR	ESDTGLAPSH	..	LWDLIMGDRQR	LGEEHPLQVA	RCTKII	KGNG	120
pombe	KRINBTVGIR	ESDTGLAPIS	..	FWDVVAADRQR	MSSEEQPLQVA	RCTKII	..	104
human	NAD	SEDPKYIINV	KQFAKFFVVDL	SDQVAPTDDIE	EGMRVGVDRN	145
rat	NAD	SEDPKYIINV	KQFAKFFVVDL	SDQVAPTDDIE	EGMRVGVDRN	145
xenopus	NAD	SEDPKYIINV	KQFAKFFVVDL	SDQVAPTDDIE	EGMRVGVDRN	145
mouse	NAD	SEDPKYIINV	KQFAKFFVVDL	SDQVAPTDDIE	EGMRVGVDRN	145
celegans	TSD	KHDFRYLINV	KQFAKFFVVDL	ADSVAPTDDIE	EGMRVGVDRN	147
cerevisiae	NNSGNSNSNS	NQQSTDADE	..	DEDAKYVINL	KQIAKFFVGL	GERVSPPTDIE	EGMRVGVDRS	180
pombe	ENQS	AEKNAYVINL	KQIAKFFVSL	GERVSPPTDIE	EGMRVGVDRN	149
human	KYQIHIFLPP	KIDPTVTMMQ	..	VEEKFPDVTYS	DVGGGCKEQIE	KLREVVETPL	LHPERFVNLG	205
rat	KYQIHIFLPP	KIDPTVTMMQ	..	VEEKFPDVTYS	DVGGGCKEQIE	KLREVVETPL	LHPERFVNLG	205
xenopus	KYQIHIFLPP	KIDPTVTMMQ	..	VEEKFPDVTYS	DVGGGCKEQIE	KLREVVETPL	LHPERFVNLG	205
mouse	KYQIHIFLPP	KIDPTVTMMQ	..	VEEKFPDVTYS	DVGGGCKEQIE	KLREVVETPL	LHPERFVNLG	205
celegans	KYQIHIFLPP	KIDPTVTMMQ	..	VEEKFPDVTYS	DVGGGCKEQIE	KLREVVETPL	LHPERFVNLG	205
cerevisiae	KYNIELPLPP	RIDPSVTMMT	..	VEEKFPDVTYS	DVGGGCKEQIE	KLREVVETPL	LHPERFVNLG	207
pombe	KYATQLPLPP	KIDPSVTMMQ	..	VEEKFPDVTYG	DVGGGCKEQIE	KLREVVETPL	LHPERFVNLG	240

Figure 3.11 (legend overleaf)

human	IEPPKGVLLF	GPPGTGKTLG	ARAVANRTDA	CFIRVIGSEL	VQKYVGEGAR	MVRELFEMAR	265
rat	IEPPKGVLLF	GPPGTGKTLG	ARAVANRTDA	CFIPVIGSEL	VQKYVGEGAR	MVRELFEMAR	265
xenopus	IEPPKGVLLF	GPPGTGKTLG	ARAVANRTDA	CFIRVIGSEL	VQKYVGEGAR	MVRELFEMAR	265
mouse	IEPPKGVLLF	GPPGTGKTLG	ARAVANRTDA	CFIRVIGSEL	VQKYVGEGAR	MVRELFEMAR	265
celegans	IEPPKGVLLY	GPPGTGKTLG	ARAVANRTDA	CFIRVIGSEL	VQKYVGEGAR	MVRELFEMAR	267
cerevisiae	IDTPPKGILLY	GPPGTGKTLG	ARAVANRTDA	TFIRVIGSEL	VQKYVGEGAR	MVRELFEMAR	300
pombe	IDPPKGIIMLY	GPPGTGKTLG	ARAVANRTDA	TFIRVIGSEL	VQKYVGEGAR	MVRELFEMAR	269
human	TKKACLIFFFD	EIDAIGGARF	DDGAGGDNNEV	QRTMLELINQ	LDGFDPRGNI	KVLMATNRPD	325
rat	TKKACLIFFFD	EIDAIGGARF	DDGAGGDNNEV	QRTMLELINQ	LDGFDPRGNI	KVLMATNRPD	325
xenopus	TKKACLIFFFD	EIDAIGGARF	DDGAGGDNNEV	QRTMLELINQ	LDGFDPRGNI	KVLMATNRPD	325
mouse	TKKACLIFFFD	EMDAIGGARF	DDGAGGDNNEV	QRTMLELINQ	LDGFDPRGNI	KVLMATNRPD	325
celegans	TKKACLIFFFD	EIDAVGGARF	DDGAGGDNNEV	QRTMLELINQ	LDGFDPRGNI	KVLMATNRPD	327
cerevisiae	TKKACIIFFFD	EIDAVGGARF	DDGAGGDNNEV	QRTMLELITQ	LDGFDPRGNI	KVMFATNRPN	360
pombe	TKKACIIFFFD	EIDAIGGARF	DDGAGGDNNEV	QRTMLELITQ	LDGFDPRGNI	KVLFATNRPN	329
human	TLDPALMRPG	RLDRKIEFSL	PDLEGORTHIF	KIHARSMSEVE	RDIRFELLAR	LCPNSTGAEI	385
rat	TLDPALMRPG	RLDRKIEFSL	PDLEGORTHIF	KIHARSMSEVE	RDIRFELLAR	LCPNSTGAEI	385
xenopus	TLDPALMRPG	RLDRKIEFSL	PDLEGORTHIF	KIHARSMSEVE	RDIRFELLAR	LCPNSTGAEI	385
mouse	TLDPALMRPG	RLDRKIEFSL	PDLEGORTHIF	KIHARSMSEVE	RDIGPELLAR	LCPNSTGAEI	385
celegans	TLDPALMRPG	RLDRKIEFSL	PDLEGORTHIF	KIHAKQMSVE	RDIRFELLAR	LCPNSTGAEI	387
cerevisiae	TLDPALMRPG	RIDRKVEFSL	PDLEGRAHIL	RTHSKMSVE	RDIRFELLAR	LCPNSTGAEI	420
pombe	TLDPALMRPG	RIDRKVEFSL	PDLEGRAHIL	RTHSKMSVE	RDIRFELLAR	LCPNSTGAEI	389
human	RSVCTEAGMF	AIRARRKIAAT	EKDFLEAVNK	VIKSYAKFSA	TPRYMTYN	433	
rat	RSVCTEAGMF	AIRARRKIAAT	EKDFLEAVNK	VIKSYAKFSA	TPRYMTYN	433	
xenopus	RSVCTEAGMF	AIRARRKIAAT	EKDFLEAVNK	VIKSYAKFSA	TPRYMTYN	433	
mouse	RSVCTEAGMF	AIRARRKIAAT	EKDFLEAVNK	VIKSYAKFSA	TPRYMTYN	433	
celegans	RSVCTEAGMF	AIRARRKIAAT	EKDFLEAVNK	VVEGYAKFSA	TPRYMTYN	435	
cerevisiae	RSVCTEAGMF	AIRARRKIAAT	EKDFLEAVNK	VISGYKQFSS	TSHYMQYN	468	
pombe	RSVCTEAGMF	AIRARRKIAAT	EKDFLEAVNK	VVKGNQKFS	TADYMNMS	438	

Figure 3.11 Comparison of peptide sequences of homologues of *S.pombe* Aps1p.

Sequences were fetched from the SWISSPROT database and aligned using the programs LINEUP and PRETTYBOX. Identical residues at any position are shown in red, functionally similar residues at that position are shown in orange, and structurally similar residues or those with the same charge, are shown in yellow. Residue numbers are shown on the right hand side. Accession numbers:- Human-P35998, Rat-Q63347, *Xenopus*-P46472, Mouse-P46471, *C.elegans*-Q18787, *S.cerevisiae*

between Snf1p and the products of *mts2*⁺, *let1*⁺ and MSS1. In addition, whereas pAS-MSS1 interacts with pACT-*mts2*⁺, it does not appear to interact with pACT-*let1*⁺.

3.6 Discussion

The use of the yeast 2-hybrid system has enabled isolation of *let1*⁺ and *aps1*⁺ as genes which can interact with *mts2*⁺ in the yeast 2-hybrid system. These genes are the *S.pombe* homologues of the *S.cerevisiae* *SUG1/CIM3* and *CIM5* genes respectively. Paradoxically, despite *CIM3* and *SUG1* being identical, *SUG1* was originally isolated as a suppressor of a deletion in a transcriptional activator (Swaffield *et al.*, 1992) and subsequently as part of a transcriptional complex (Kim *et al.*, 1994). By contrast, both *CIM3* and *CIM5* were isolated in a different screen to look for targets of the Cdc28 kinase (Ghislain *et al.*, 1993). The *cim3-1* and *cim5-1* mutants were found to be lethal when in combination with the *cdc28-1N* mutation, suggesting that activation of the 26S proteasome may be due to the Cdc28 protein kinase.

The predicted protein encoded by *let1*⁺ is highly homologous (73% identity) to the CAD region of a family of proteins, the AAA family, of which Mts2p, MSS1p and Aps1p are also members (Confalonieri and Duguet, 1995). Recent work has shown that Sug1p is a subunit of the proteasome in budding yeast (Rubin *et al.*, 1996). Furthermore antibodies raised against both this subunit and against Cim5p, cross-react with subunits of the 26S proteasome in extracts from *S.pombe* (W.Dubiel, unpublished result, and see Figure 5.11 panel B and C). Thus, like Mts2p and MSS1p, Let1p and Aps1p may be subunits of the 26S proteasome in *S.pombe*.

Of 50 cDNAs characterised in this screen, 41 encode the *S.pombe let1*⁺ gene and 6 encode *aps1*⁺, the *S.pombe* homologue of *CIM5* and MSS1. As yet, no genetic interaction has been demonstrated between Mts2p and Let1p. The interaction between Mts2p and the putative protein encoded by MSS1, the murine homologue of *CIM5* and *aps1*⁺, has been demonstrated genetically, since overexpression of MSS1 can rescue the *ts* defect of *mts2-1*, but cannot rescue a null allele of *mts2* (Gordon *et al.*,

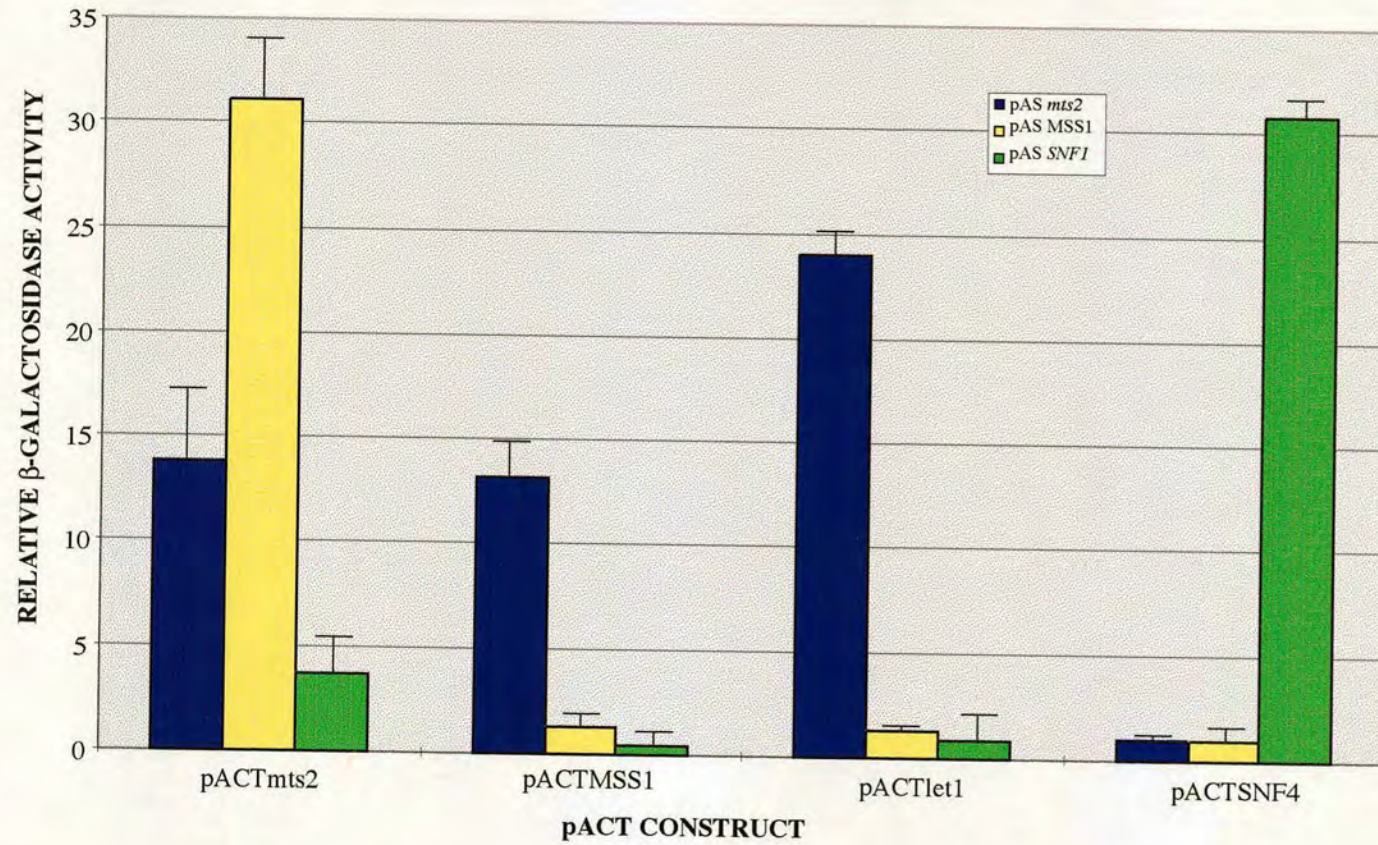


Figure 3.12 2-hybrid interaction between ATPase subunits

Different combinations of pAS and pACT constructs were co-transformed into *S.cerevisiae* Y190 cells and tested for their β -galactosidase activity in liquid assay as before. Activity is shown relative to the interaction between the pAS construct and pACT plasmid alone.

1993). This reflects a requirement for the presence of the mutant protein encoded by *mts2-1* for suppression by MSS1 to take place.

The product of MSS1 did not interact with the product of the *let1⁺* gene in the 2-hybrid system. Given the model for the formation of an ATPase 'ring' within the 19S regulatory complex (Dubiel *et al.*, 1993), one interpretation of this result is that the subunit encoded by *mts2⁺* lies between Let1p and the *S.pombe* homologue of MSS1p (see Figure 7.1). This being so, it might be expected that these three ATPases might form half of the ATPase 'ring', the other half being made up of TBP1p, TBP7p and Sug2p. Another interpretation of the 2-hybrid results is that the murine S7 protein is too dissimilar from the *S.pombe* S7 protein. This is clearly unlikely since the mouse MSS1 gene can rescue a deletion of *CIM5*, its budding yeast homologue (Ghislain *et al.*, 1993). Secondly a comparison between protein sequences of MSS1 homologues from 7 different species (Figure 3.11) shows a very high homology in all regions of the protein. The full length *aps1⁺* gene requires to be cloned and tested for suppression of the *mts2-1* mutant.

The failure to isolate non-ATPase subunits of the 26S proteasome in this screen is surprising. Overexpression of *mts2⁺* has been shown to suppress the phenotype of *mts4-1* at 32°C but not at 36°C. This suggests that Mts4p binds Mts2p *in vivo*, and has been backed up by a demonstration of synthetic lethality between *mts2-1* and *mts4-1*. In addition, an *in vitro* assay has demonstrated binding of Mts2p and Mts4p (Wilkinson *et al.*, 1997, in press). This is the first evidence for a genetic interaction between an ATPase and a non-ATPase subunit of the 19S cap complex. Furthermore, Mts2p and Mts4p have been shown to bind in the 2-hybrid system, indicating that this screen has not been saturated. It might therefore be expected that the *mts4⁺* gene would be isolated in a more exhaustive 2-hybrid screen.

Just as the search for suppressors of *mts2-1* failed to uncover anything other than *mts2⁺*, perhaps the conditions for detecting interactions between ATPases and non-ATPases in this 2-hybrid screen were too stringent. For example, only large colonies were selected during the initial screen. Perhaps smaller colonies might have con-

tained genes other than *let1*⁺ and *aps1*⁺, which interacted less strongly. If this was the case, it would provide a correlation between colony size and strength of interaction.

The sequence of the insert contained within 2H10 was found to be identical to that of a cosmid currently being sequenced in the *S.pombe* sequencing project. Translation of this sequence revealed a predicted polypeptide of around 119kDa. Although the largest subunit of the 19S regulatory complex, S1, has a molecular weight of 112kDa, is possible that the protein encoded by 2H10 may represent an as yet unidentified regulatory subunit. The N-terminus of this ORF, in common with S12 of the 19S cap complex, contained large stretches of lysine and glutamic acid residues. Although these KEKE motifs have no function as yet assigned to them, they have been hypothesised to mediate protein-protein interactions and to be involved in the presentation of peptides by MHC Class-I receptors (Realini *et al.*, 1994). Further experiments are required to test whether this protein is indeed present in the 26S proteasome of *S.pombe*.

In conclusion, genes encoding two members of the AAA family, *let1*⁺ and *aps1*⁺, that interact with Mts2p, have been isolated using the yeast 2-hybrid system. Homologues of both genes have now been shown to encode subunits of the 26S proteasome (Ghislain *et al.*, 1993; Rubin *et al.*, 1996), suggesting that these proteins may interact in this complex *in vivo*. It is possible that they form part of an ATPase ring structure which would facilitate passage of the target protein through the 19S complex into the catalytic chamber of the 20S complex. Other ATPase subunits of the 26S proteasome are thought to complete this ring structure. The interactions between pairs of these subunits are consistent with this model (Ohana *et al.*, 1993; Russell *et al.*, 1996; Choi *et al.*, 1996).

Chapter 4 Localisation of the interaction between Let1p and Mts2p

4.1 Introduction

The yeast 2-hybrid system was successfully used for the isolation of *aps1*⁺, the *S.pombe* homologue of the mouse MSS1 gene, and *let1*⁺, the *S.pombe* homologue of the budding yeast *SUG1* gene. Based on the rescue of the *ts* phenotype of *mts2-1* by over-expression of the mouse MSS1 gene, the isolation of *aps1*⁺ by a 2-hybrid screen might have been predicted. However, the isolation of *let1*⁺ was unexpected. This was because the protein encoded by *SUG1* was thought to be involved in regulation of the expression of the budding yeast Gal4 transcriptional activator (Swaffield *et al.*, 1992). Homologues of the proteins encoded by *let1*⁺ and *aps1*⁺ have since been shown to be identical to subunits S7 and S8 of the 26S proteasome respectively (Dubiel *et al.*, 1993; Dubiel *et al.*, 1995; Rubin *et al.*, 1996).

The 6 proteasomal ATPases are predicted to adopt a ring conformation which may contact the 20S catalytic core of the 26S proteasome (Hershko and Ciechanover, 1992). The results from interaction studies between Mts2p, MSS1p and Let1p (see section 3.5) lend support to this model. Although MSS1p (S7) interacts with Mts2p (S4), it does not interact with Let1p (S7). Mss1p may therefore be physically separated from Let1p (S8), by Mts2p (S4). Although the lack of interaction between MSS1p and Let1p may have been due to the use of the murine S7 subunit instead of the *S.pombe* S7 subunit this is probably not the case for the reasons outlined below: A comparison of peptide sequences of S7 family members reveals a high level of identity (see Figure 3.11). In addition, over-expression of the mouse MSS1 gene can rescue a deletion of its budding yeast homologue (Ghislain *et al.*, 1993). This ordered interaction then may reflect the *in vivo* situation between proteasomal ATPases, thereby giving clues as to their position within the ATPase ring.

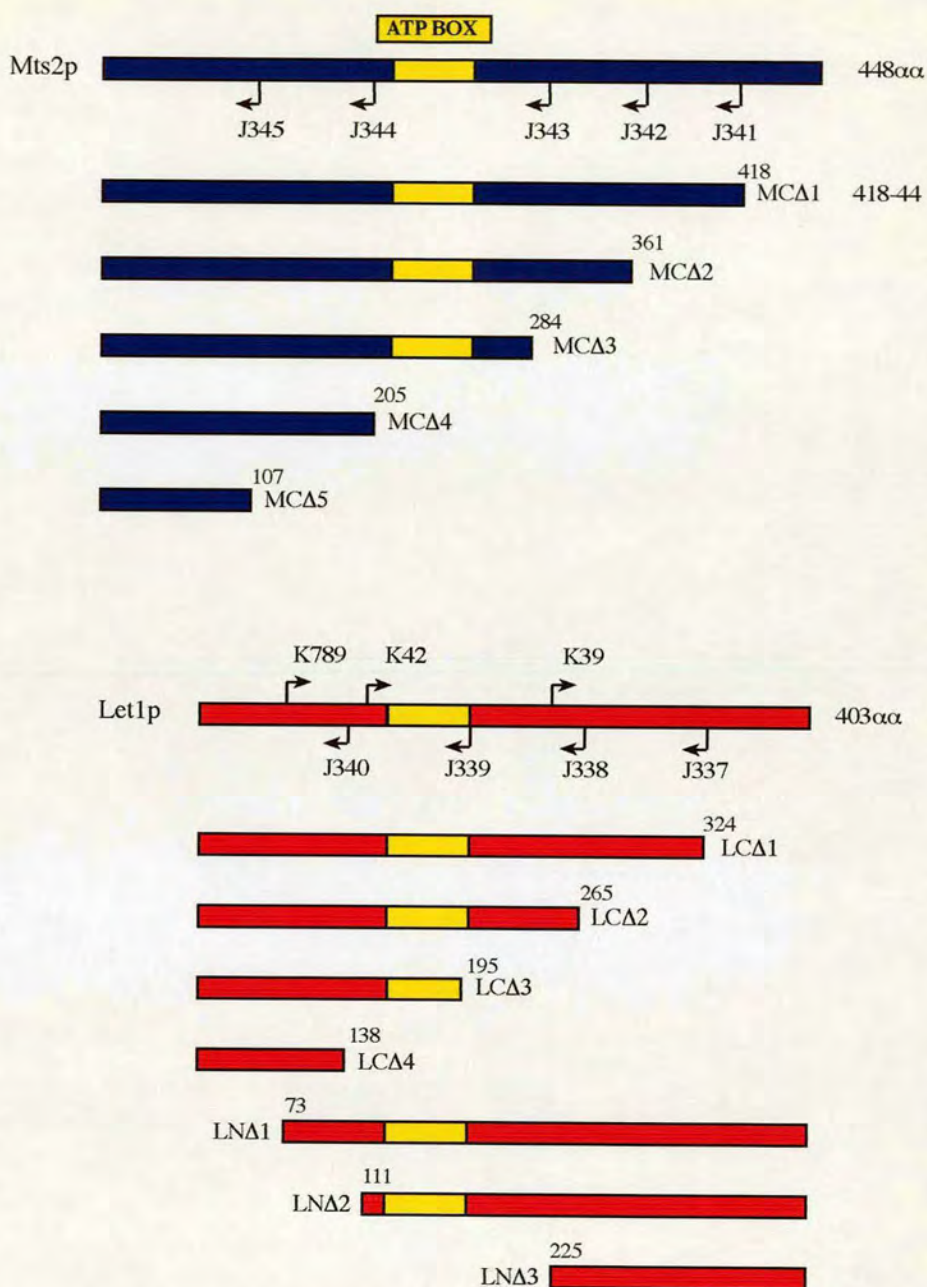


Figure 4.1 Strategy for generation of truncations in the Mts2p and Let1p ORF

Nested oligonucleotide primers, complementary to the *mts2⁺* and *let1⁺* genes were designed. The position of the primers relative to the gene and to the ATP-binding box, is shown. The sizes of the putative truncations, in amino acid residues, is shown above the truncations. Truncations were named according to the size of the deletion, and the end from which the deletion was made, thus, MCA1 is the smallest truncation from the C-terminus of Mts2p.

All of the 26S proteasomal ATPases contain a conserved ATPase domain (CAD) of around 200 amino acid residues in length. The alignment of these ATPases is shown in Figure 1.3. The 73% degree of identity of this region between these proteins is striking. By contrast, the N-termini of all of these proteins bear little resemblance to each other suggesting that for each molecule, this domain might impart some form of specificity either in intra- or extra-proteasomal interactions.

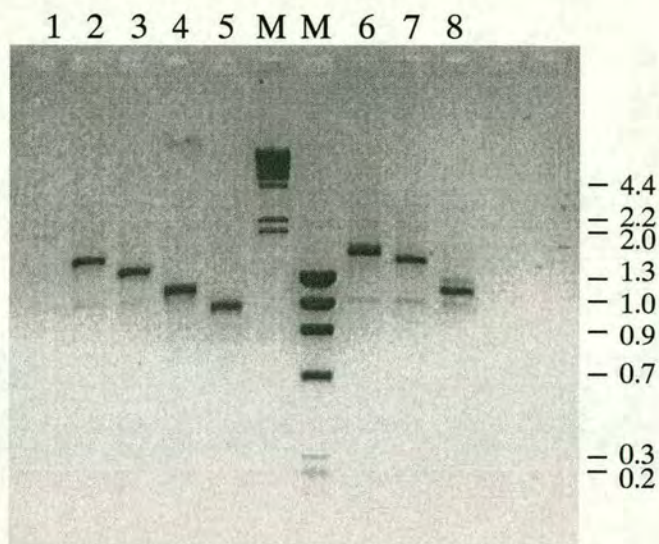
At least three of the proteasomal ATPases, TBP-1, TBP-7 and Sug1p, contain a leucine zipper-like motif in the N-terminus (Ohana *et al.*, 1990; Wang *et al.*, 1996). The leucine-zipper motif is known to mediate protein-protein interactions (Johnson and McKnight, 1989), and in the case of Sug1p, is predicted to form a coil-coil interface which facilitates its interaction with the nuclear transcription factor c-Fos (Wang *et al.*, 1996). The N-termini of TBP-1 and TBP-7 were shown to be necessary and sufficient for their heterodimerisation *in vitro* (Ohana *et al.*, 1993). This indicated that in addition to facilitating interactions with proteasomal substrates such as c-Fos, the N-termini of the proteasomal ATPases might interact during formation of the putative ATPase ring *in vivo*.

As a means of testing the above hypothesis, the interaction between Let1p and Mts2p was investigated. In addition, since, from the results of the interaction studies in Chapter 3, Mts2p interacted with itself, this interaction was also studied. Truncations of both Mts2p and Let1p were made and expressed in the 2-hybrid system. The results of these experiments, and those which assay the effect of over-expressing truncations of these proteins *in vivo*, are described in this chapter.

4.2 Construction of deletions in Mts2p and Let1p

Nested oligonucleotide primers were used to generate N-terminal and C-terminal deletions of the *mts2⁺* and *let1⁺* genes by PCR. The strategy used is shown in Figure 4.1, and the sequence and position of the primers used is shown in Appendix A. In order to identify the region of the proteins responsible for interaction, increasing amounts of the respective ORFs were removed. The nomenclature of these deletions

A



B

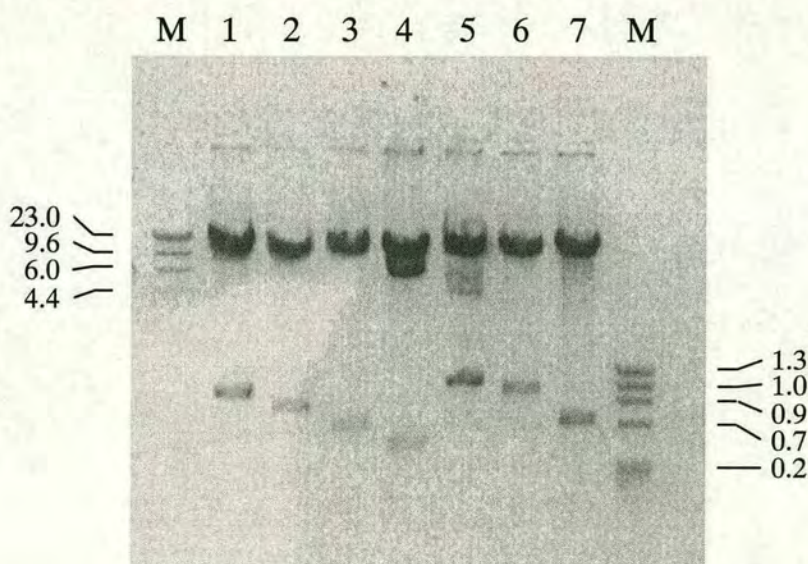


Figure 4.2 Cloning of PCR-generated deletions of *let1* into pACTII

(A) 5µl of PCR products from a 50µl PCR reaction, generated using the oligonucleotides shown in Figure 4.1, were run on a 0.8% TAE gel. Lane 2-5, LCA1-LCA4. Lane 6-8, LNA1-LNA3. Full length *let1*⁺ cDNA is shown in Lane 1. (B) The PCR products from Lanes 2-8 in A were cleaned, digested to completion with *Nco*I and *Xho*I, and cloned into the vector pACTII (see Figure 2.4). Once cloned, 1µg of the constructs were re-digested with the above restriction enzymes and run on a 0.8% TAE gel. (Lanes in B)

reflects the size of the deletion and the end from which the ORF was deleted. MCA1 is the smallest deletion from the C-terminus of Mts2p, whereas LNΔ3 is the largest deletion from the N-terminus of Let1p. The truncations were cloned into the vector pACTII (see Materials and Methods). The results of the PCR and cloning of deletions of the *let1*⁺ gene are shown in Figure 4.2. Following cloning of the PCR fragments, the constructs were completely sequenced to ensure that no PCR errors had been generated.

4.3 Expression of truncated proteins

In order to detect expression of the truncated proteins in yeast cells, it is possible to perform a Western blot using a commercially available antibody raised against the Gal4 activation-domain epitope (Clontech 5398-1). Protein extracts were made from yeast cultures, separated on a poly-acrylamide gel, and western blotted. Although this experiment was repeated several times, no cross-reaction was observed. However, while investigating *in vitro* binding of Mts2p to truncations of Let1p, the constructs were cloned into the vector pET6H and expressed by *in vitro* translation. Expressed proteins of the correct molecular weight were clearly visible (data not shown).

4.4 Analysis of 2-hybrid interactions

S.cerevisiae Y190 cells which had been co-transformed with pAS-*mts2*⁺ (full length) and the truncated *let1* constructs LCΔ1-LCΔ4 and LNΔ1 in pACTII, were tested for their ability to interact on both solid and in liquid media. In this experiment, 4 independent isolates from each construct combination were tested, and the strength of the interaction was quantitated as before (see Chapter 3). The results of this experiment are shown in Figure 4.3. All levels of activity are expressed as a percentage of the interaction between full length molecules.

MCA1, carrying the smallest C-terminal truncation, produced more than half of the β galactosidase activity of full length Mts2p. This was restored by further truncations

<u>pAS construct ‘bait’</u>	<u>pACT-construct ‘target’</u>	<u>Growth on 3-AT</u>
<i>mts2</i> ⁺	<i>let1</i> ⁺	++++
	LCΔ1	+
	LCΔ2	++
	LCΔ3	+
	LCΔ4	+++
	<i>SNF4</i>	-
MCA1	<i>let1</i> ⁺	++++
	LCΔ1	-
	LCΔ2	++
	LCΔ3	+
	LCΔ4	+++
	<i>SNF4</i>	-
MCA3	<i>let1</i> ⁺	++++
	LCΔ1	-
	LCΔ2	++
	LCΔ3	+
	LCΔ4	++
	<i>SNF4</i>	-
MCA4	<i>let1</i> ⁺	++++
	LCΔ1	-
	LCΔ2	++
	LCΔ3	+
	LCΔ4	++
	<i>SNF4</i>	-
MCA5	<i>let1</i> ⁺	++++
	LCΔ1	-
	LCΔ2	+
	LCΔ3	-/+
	LCΔ4	++
	<i>SNF4</i>	-

Table 4.1 Interaction of Mts2p with Let1p following nested C-terminal truncations of both ORFs. Y190 cells co-transformed with both constructs were plated on media containing 3-AT. Results were based on the growth of cells relative to the full length control. PACT-*SNF4* was used as a negative control.

until the construct MCA4, which removes all of the ATP-binding motif, was tested. When this happens, two thirds of the activity is lost, indicating that for an interaction between Mts2p and itself the region containing the ATP-binding motif may be important.

By contrast to the above, the interaction between full length Mts2p and truncations of Let1p produce different results. Again, as is the case with deletions of Mts2p, C-terminal truncations seem to have little or no effect, even when only the N-terminal 137 amino acids remain. When the N-terminal 72 amino acids are removed, in the construct LNA1, however, the relative activity decreased, suggesting that the region between amino acids 73 and 138 is involved in mediating the interaction between the 2 subunits.

The C-terminal truncations CA1-CA5 of Mts2p were then cloned into the vector pAS2 and tested with the C-terminal truncations of Let1p in the vector pACTII. This time, colonies were tested for their ability to grow on media containing 3-AT. The results of this are shown in Table 4.1. Significantly, it appears that on this media the Mts2p truncation MCA5, which removes all but 108 N-terminal amino acids, can interact with both full length and C-terminally truncated versions of Let1p. By contrast, there was no growth of colonies when MCA5 was co-transformed with the negative control, pACT-SNF4.

Although these interactions remain to be quantified by liquid assay, they support the results of the earlier experiments which suggested that the N-termini of both Mts2p and Let1p, are important for an interaction between full length molecules. The region between amino acids 73-138 in Let1p are involved in this interaction.

The interaction between full length Mts2p in pAS1 and truncated versions of Mts2p in pACTII, reflect a requirement for a region of the protein which contains all or part of the ATP-binding cassette. This suggest that homodimerisation of Mts2p is mediated by a region distinct from that involved in heterodimerisation between Mts2p and Let1p.

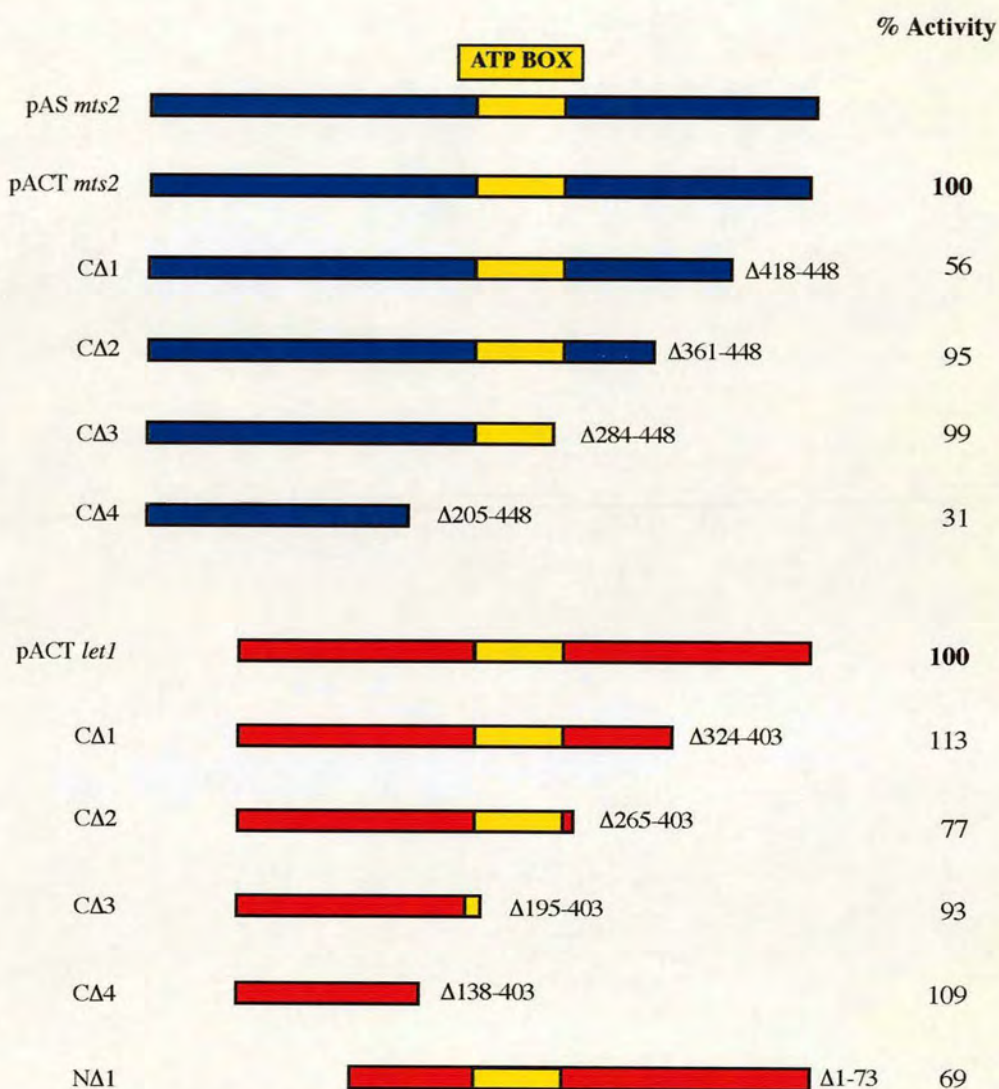


Figure 4.3 Quantitation of the 2-hybrid interaction between pAS-*mts2*⁺ and truncated forms of the *mts2* and *let1* genes.

S.cerevisiae Y190 cells were co-transformed with pAS-*mts2*⁺ and individual truncations of the *mts2* and *let1* ORF. The activity was measured as a percentage of the interaction between full length gene products. LNA2 and LNA3 were not used in this experiment. The activity produced by interaction of pAS-*mts2*⁺ with pACT-*SNF4* and of pAS-*SNF1* with all of the constructs was also measured (not shown). The ATP box contains the Walker A + B nucleotide-binding motifs (Walker *et al.*, 1982)

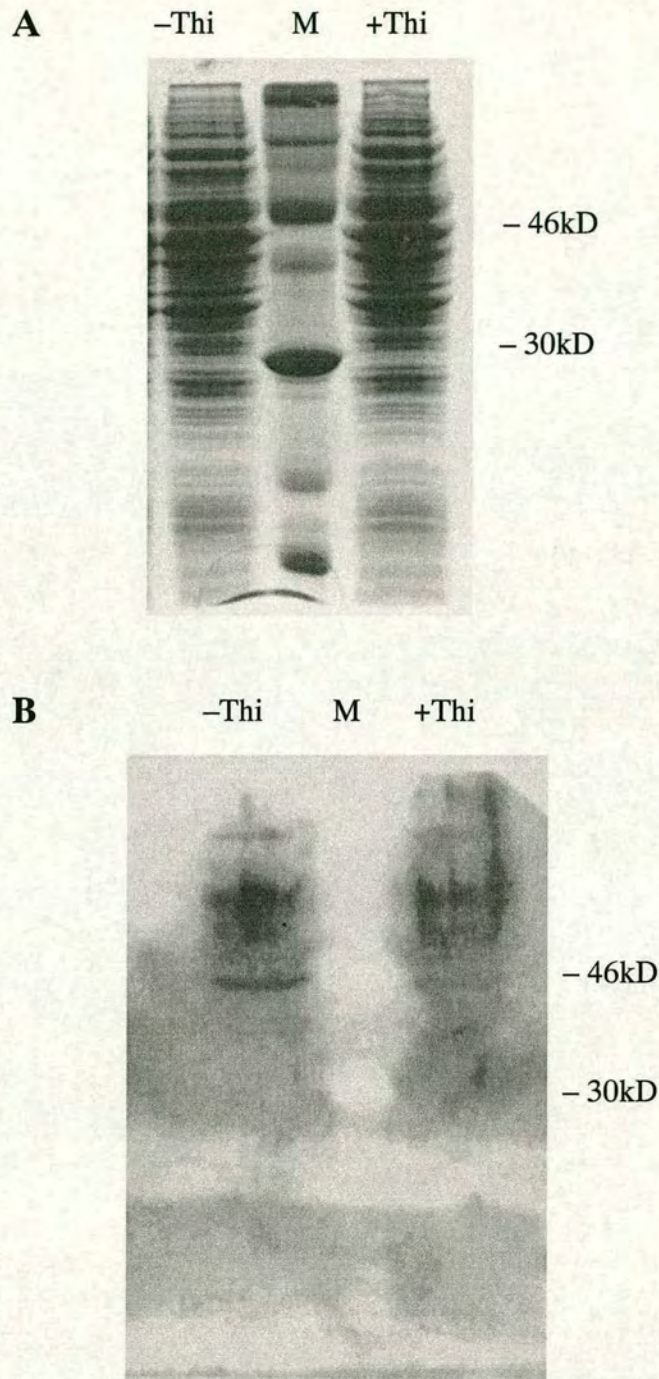


Figure 4.4 Overexpression of *let1*⁺ in pREP1

Protein extracts were made from *S.pombe* cells, carrying the construct pREP-*let1*⁺, grown in the presence (+Thi) and absence (-Thi) of 4M thiamine. 10µg of total cell protein was loaded, along with markers, on each of 2 10% polyacrylamide gels. One gel (A) was stained with coomassie brilliant blue. Protein from the second gel (B) was transferred onto nitrocellulose and probed with anti-Let1p antibodies (1:10 000 dilution in 'Blotto'). The position of the 30kDa and 46kDa protein markers are shown.

4.5 Expression of Let1p in *S.pombe*.

The previous experiments suggested that the interaction of Mts2p and Let1p was mediated by sequences within the N-termini of both proteins. If this was so, then the over-expression of truncated Let1 molecules which had had their N-termini removed, may have little or no effect on cell viability. However, by analogy to the result when truncations of Mts2p were assayed (L. Colleaux, unpublished result), over-expression of C-terminally truncated forms of Let1p might have a dominant negative effect because they still possessed the capacity to interact with other 26S proteasome subunits. In order to study this, truncations of the *let1*⁺ gene, shown in Figure 4.1, were cloned into the *S.pombe* expression vector pREP1, and expressed *in vivo*.

4.5.1 Expression of full length Let1p

Full length *let1*⁺ was cloned into pREP1 and expressed *in vivo* under the control of the *nmt1* promoter. Analysis of the expression of this construct was facilitated by the purification of polyclonal anti-Let1p antibodies from rabbit which had been raised against recombinant GST-*let1*⁺ (made by C. Wilkinson). The results of this experiment are shown in Figure 4.4. Figure 4.4 (B) shows the expression of full length Let1p from this construct in the presence (+) and absence (-) of thiamine. The polyclonal antibodies recognise a band of approximately 45kDa. This is in agreement with the observed size of the human variant of Let1p, p45 (Akiyama *et al.* 1995).

The level of expression of the cloned *let1*⁺ gene in the absence of thiamine is higher than that of the wild type level. However this is less than might have been expected, given the strength of this promoter (see Table 2.1). There are two possible explanations for this result. Firstly, there may be a mutation in the promoter causing reduced expression, as would have been expected for the vectors pREP41 or pREP81. Alternatively, some form of feedback mechanism may be acting to ensure that non-stoichiometric levels of Let1p are not produced.

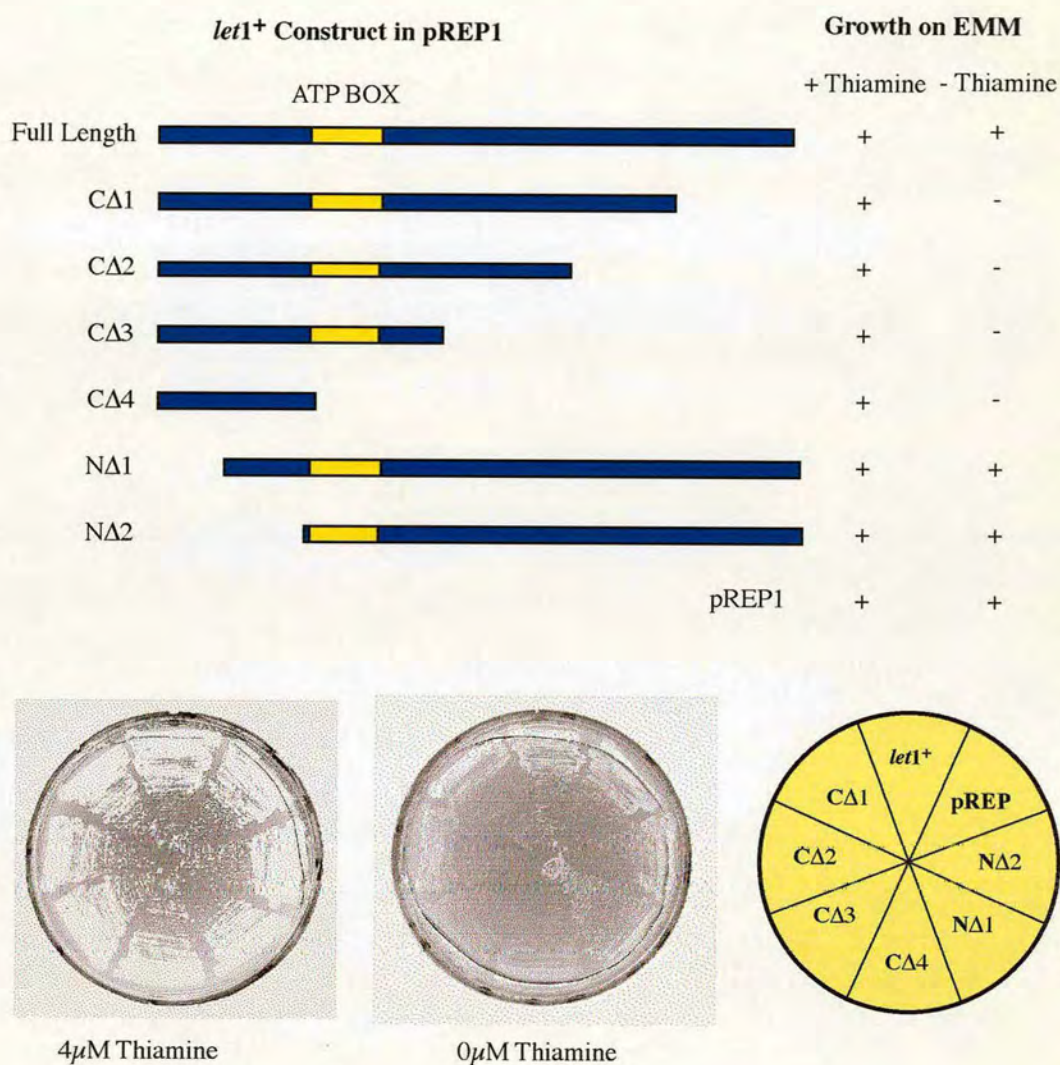


Figure 4.5 Overexpression of *let1* deletions *in vivo*

Full length *let1*⁺, along with the deletion constructs LCA1-LCΔ4 and LNΔ1-LNΔ2, were cloned into the expression vector pREP1 and transformed into a wild type strain of *S.pombe*. Cells which had been transformed with these constructs were streaked onto EMM plates with and without 4M thiamine, and incubated at 30°C.

4.5.2 Expression of truncated forms of Let1p

The truncations LCA1-LCA4 and LNA1-LNA2 were sub-cloned into pREP1. The constructs, along with full length *let1*⁺ and pREP1 as controls, were transformed into a wild type *leu1-32 h*⁻ strain and plated on media which contained 4μM thiamine. Once grown, the colonies from each transformation were picked and streaked onto two EMM plates, only one of which contained thiamine. The result of these experiments is shown in Figure 4.5. It is quite clear that over-expression of neither LNA1 or LNA2 is deleterious. However, over-expression of all of the C-terminal truncations, LCA1-LCA4, is lethal.

This dominant negative effect caused by over-expression of C-terminal truncations of Let1p supports the results obtained when a C-terminally truncated Mts2p (pSP31) was over-expressed (L. Colleaux, unpublished results). In the case of pSP31, the lethal phenotype was rescued by over-expressing full length Mts2p. In addition, an over-expression of MSS1, the mouse homologue of *aps1*⁺, could also rescue the dominant lethality of the over-expression of pSP31. This result may have been expected, given the way in which MSS1 had been isolated as a suppressor of *mts2-1*, and confirmed the genetic interaction between these ATPase subunits of the 26S proteasome (Gordon *et al.*, 1993).

4.6 Discussion

The above experiments describe an analysis of the interaction between 2 proteasomal ATPases, Mts2p and Let1p, and the effect of over-expressing these truncations *in vivo*. The results indicate that the products of the *mts2*⁺ and *aps1*⁺ genes interact *in vitro* in the 2-hybrid system and that this interaction is mediated by the N-termini of both proteins. By contrast the interaction between Mts2p and itself appears to be mediated by a distinct region of the polypeptide which includes the ATPase domain. Over-expression of the non-conserved N-terminal domain of Let1p *in vivo* is lethal.

This dominant lethality may occur because this region is responsible for inter-subunit interaction during 26S proteasome complex formation.

A number of interactions involving homologues of Let1p have been described. Using a 2-hybrid screen, the human homologue of Let1p, Sug1p, has been shown to interact with the retinoic acid receptor in a ligand-dependent manner. This interaction was mediated by an N-terminally truncated (amino acid residues 1-149) version of SUG1p, (vom Baur *et al.*, 1996). This molecule lacked the N-terminal coiled-coil region, but retained the ATP-binding motif. This work also demonstrated an interaction between the ATPase domain of SUG1p and the activation domain of the transcription factor AF-2. By contrast, other work has shown that FZA-B, a mammalian homologue of Let1p, interacts with the transcription factor c-Fos, and that this interaction is mediated by the predicted leucine-zipper of FZA-B and the bZIP segment of c-FOS (Wang *et al.*, 1996). It is therefore possible that Let1p can interact with other protein molecules either via the conserved CAD domain, or by the non-conserved N-terminal region.

How can Sug1p / Let1p use the same non-conserved region to interact both with other proteasomal ATPase subunits and also with transcription factors? It is not inconceivable that Let1p has more than one function. That is, it may function to recruit substrates of the 26S proteasome, of which c-Fos is one (Stancovski *et al.*, 1995), as well as binding other proteasomal ATPases such as Mts2p. The manner in which this might occur is unknown, but it is possible that an internal signal such as the change in phosphorylation state of Let1p or a binding partner, might be required both for the association of Let1p with target substrates and with other proteasomal subunits. Phosphorylation of c-Jun, the binding partner of c-Fos, increased degradation of c-Fos by the 26S proteasome (Stancovski *et al.*, 1995). Sug1p itself is also found in a phosphorylated state (Sato *et al.*, 1995). Therefore, a change in conformation elicited by a change in the phosphorylation state of Let1p / Sug1p may enable the simultaneous binding of substrates and other proteasomal subunits.

There have been suggestions that these proteasomal ATPases are 'sticky', thus reflecting the observation of non-specific interactions or two-hybrid 'false positives

(Hengen, 1997). However, given the dominant negative phenotype of a C-terminally truncated Let1p molecules versus the ineffectual N-terminal truncations, it is difficult to argue against the interaction of the N-terminus of Let1p with another molecule. From the results of Chapter 3, this may be another proteasomal ATPase. The absence of this region of the molecule, in this model, would preclude the formation and thus function of the 26S proteasome.

In summary, then, it has been shown that the N-terminus of 2 proteasomal ATPases are necessary for heterodimerisation. In contrast, homodimerisation of Mts2p required an intact ATP-binding domain. This situation is similar to that observed for interactions between the human homologue of Let1p and the nuclear retinoic acid receptor α (RAR α). Over-expression of a C-terminally truncated form of Let1p has a dominant negative effect. This is possibly due to the inability of cells to form a functional 26S proteasome.

Chapter 5

Chapter 5 Analysis of a *let1*⁺ disruption

5.1 Introduction

The cDNAs encoding Let1p and Aps1p were isolated on the basis of their interaction with Mts2p in the yeast 2-hybrid system. Homologues of both Let1p and Aps1p have been shown to be components of the 26S proteasome. The peptide encoded by MSS1, the murine homologue of *aps1*⁺, was first isolated as a suppressor of a mutation in a protein kinase (Irie *et al.*, 1989). It has been shown to be identical to subunit 7 of the human erythrocyte 26S proteasome (Dubiel *et al.*, 1993). *SUG1*, the budding yeast homologue of *let1*⁺, was isolated as a suppressor of a C-terminal deletion in the Gal4 transcription factor. Consequently it was thought to be a transcriptional regulator (Swaffield *et al.*, 1992). However, recent work has demonstrated the presence of Sug1p in purified 26S proteasome protein extracts (Rubin *et al.*, 1996).

With one exception, subunit 5a (S5a), disruption of genes encoding subunits of the regulatory complex of the 26S proteasome in budding and fission yeast has been shown to be lethal (Ghislain *et al.*, 1993; Gordon *et al.*, 1993; 1996 ; Michael *et al.*, 1994; Coux *et al.*, 1996; van Nocker *et al.*, 1996; Wilkinson *et al.*, 1997). The phenotype associated with these disruptions is similar in most cases, and results in the accumulation of cells which arrest at the metaphase stage of mitosis. For this reason it was hypothesised that if *let1*⁺ encoded a subunit of the 26S proteasome, not only would a disruption of *let1*⁺ be lethal (Michael *et al.*, 1994), but it would also result in a phenotype similar to that caused by a disruption of *mts2*⁺, *mts3*⁺ or *mts4*⁺ (Gordon *et al.*, 1993, 1996; Wilkinson *et al.*, 1997).

There are two strategies for construction of null alleles in fission yeast: insertion of a selectable marker into the middle of an open reading frame resulting in a disrupted gene, or replacement of all or a large part of the coding region of the gene with a selectable marker. In both cases, nutritional markers such as the *S.cerevisiae* *LEU2*⁺

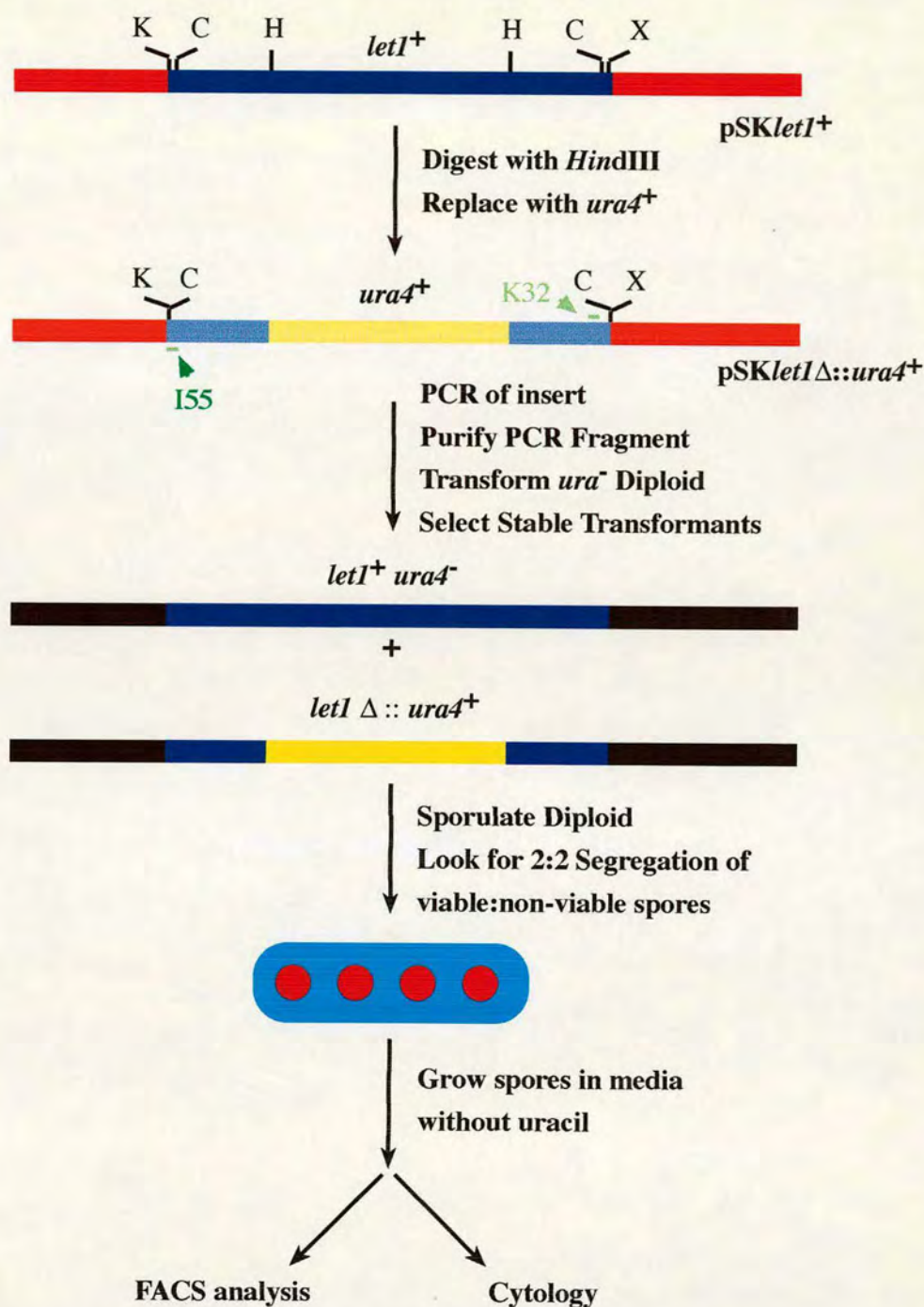


Figure 5.1 Strategy for the construction of a *let1::ura4⁺* heterozygous diploid

The *HindIII* fragment was removed and replaced by the *S.pombe ura4⁺* gene. The oligonucleotide primers I55 and K32, complementary to the *let1* genomic DNA, were then used to remove the fragment containing the *ura4⁺* gene by PCR. Following transformation and sporulation of a diploid strain carrying this construct, spores were to be used for FACS analysis and cytology. K=KpnI H=HindIII X=XbaI C=Clal

gene or the *S.pombe ura4⁺* gene are commonly used. Both approaches can be performed using a one-step gene-replacement approach (Rothstein, 1983).

Targeted integration is facilitated by leaving a variable amount of homologous DNA at either end of the resulting construct. These free ends are recombinogenic and interact directly with homologous sequences in the chromosomal DNA. The length of DNA required for efficient chromosomal integration varies greatly. Whereas 50-80 nucleotides of homology in the region adjacent to the nutritional marker can facilitate recombination, 0.3kb of homology ensures a higher recombination frequency (Rothstein, 1983).

5.2 Construction of a null allele by gene replacement

The strategy for deletion and replacement of the *let1⁺* coding region and its subsequent transformation into a diploid strain is shown (Figure 5.1). The 1.9kb genomic *ClaI* fragment containing the complete *let1⁺* gene was isolated from the vector pKO45 (pFL20) (Michael *et al.*, 1994). The fragment was amplified by PCR using oligonucleotides J165 and J166 which contained a *KpnI* and an *XbaI* site respectively. After digestion of the PCR product with these enzymes, it was purified and cloned into the *KpnI* and *XbaI* sites of pSK⁺. The resulting construct, pSK-*let1⁺*, was 4.8kb.

Replacement of the *let1⁺* gene was facilitated by the presence of two *HindIII* sites within the ORF. Digestion of the construct pSK-*let1⁺* with *HindIII* released an 0.87kb fragment containing the C-terminal 233 codons of the *let1* gene. The 1.8kb *ura4⁺* *HindIII* fragment, from the vector pIRT-*ura4⁺*, was cloned into the *HindIII* sites of pSK-*let1⁺* which had been treated with CIP to prevent recircularisation. The resulting plasmid, pSK-*let1Δ::ura4⁺*, now contained 1.1kb of genomic DNA 5' and 0.18kb 3' to the *ura4⁺* insert. The orientation of the *ura4⁺* gene into pSK-*let1* was checked by sequencing of the new construct pSK-*let1Δ::ura4⁺*. Oligonucleotide primers on either side of the site of insertion were used. The results confirmed that

the *ura4⁺* gene had inserted with the direction of transcription opposite to that of the disrupted *let1* gene (data not shown).

In order to liberate the *ura4⁺* marker and flanking *let1* DNA the new construct was digested to completion with *Cla*I. However, instead of two fragments, corresponding to the vector (2.9kb) and the disrupted *let1* genomic fragment (3.04kb), being liberated as expected, only one band (6.0kb) was visible. This 6kb band corresponded to the entire construct which appeared to have cut only once. Thus one of the *Cla*I sites had become resistant to enzymatic cleavage, possibly as a result of the PCR amplification step. Instead of remaking the construct, oligonucleotides I55 and K32, which were homologous to the N- and C-termini of the insert respectively, were used in a PCR reaction to remove the disrupted *let1* gene. The product of this reaction was a 2.48kb fragment that had 5' and 3' ends homologous to the chromosomal *let1⁺* locus. This fragment was used to transform a diploid strain to uracil prototrophy.

5.3 Isolation of stable *let1*Δ::*ura4⁺* integrants

The purified linear PCR fragment from the construct pSK-*let1*Δ::*ura4⁺* was transformed into diploid cells (see Materials and Methods). Thirty-four *ura⁺* colonies were patched onto selective media then tested for plasmid stability to check whether the PCR fragment had integrated, or whether it was being episomally maintained due to the presence of an autonomously replicating (ARS) sequence. Following this procedure, 20 *ura⁺* colonies, which seemed to have a stably integrated copy of the *ura4⁺* gene, were selected for further analysis.

5.4 Localisation of the site of integration

Although stable integration of the disrupted *let1* construct had taken place, it was important to localise the site of integration. This was done by PCR analysis and Southern blotting. The former made use of an oligonucleotide internal to the construct, and another upstream of the site of integration. The use of Southern blotting for analysing

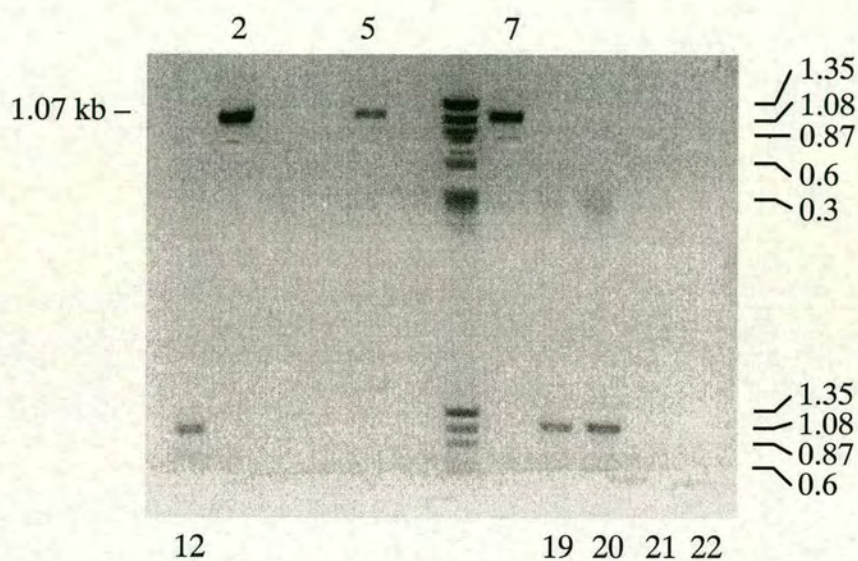


Figure 5.2 PCR of putative *let1* disruptions

Twenty stable *ura4⁺* transformants were analysed by PCR for the presence of an integrated copy of the disrupted *let1* allele. The oligonucleotide primers K250, 5' to the site of integration of the construct, and C432, complementary to the 5' end of the *ura4⁺* gene were used to amplify a diagnostic PCR product of 1.07kb. 5µl of a 50µl PCR reaction was run on a 0.8% TAE gel. Lanes 1-20 -PCR products from transformants 1-20, Lane 21 -*ura⁻* diploid, Lane 22 -linear *let1⁺* PCR fragment containing *ura4⁺* gene.

the site of integration was facilitated by knowledge of the restriction map of the *letI*⁺ chromosomal locus (Michael *et al.*, 1994).

5.4.1 PCR analysis

The results of the stability test indicated that 20 of the 34 primary isolates carried an integrated *ura4*⁺ gene and thus presumably a disrupted *letI* allele. DNA from cells carrying the potential integrants was used in a PCR reaction to check for integration of the disrupted *letI* allele at the correct chromosomal locus. One of the primers used, K250, was 5' to the PCR fragment used for integration and the other, C432, was complementary to the 5' end of the *ura4*⁺ insert. The expected size of the product was 1.07kb. A product of this size would be diagnostic for the integration of the disrupted *letI* gene at the correct chromosomal locus.

The result of this PCR reaction (Figure 5.2) shows that of the 20 colonies tested, 6 gave the correct size of PCR product. Since the controls in this experiment were negative (lanes 21 and 22) it is reasonable to assume that those which produced a positive result have integrated at the correct locus. This was directly tested by performing Southern blot analysis on DNA prepared from the 6 positive colonies.

5.4.2 Southern blot analysis of *letI*Δ::*ura4*⁺ integrants

A ³²P-labelled DNA probe complementary to the *letI*⁺ gene was used to test the result of the preceding PCR reaction. Hybridisation of this probe to DNA from diploid cells heterozygous for a disruption at the *letI*⁺ locus should result in 2 labelled bands. These bands would correspond to the wild type allele and to the disrupted allele carrying the *ura4*⁺ gene and would have a size of 4.6kb and 5.7kb respectively.

Genomic DNA was prepared from each isolate and digested overnight using the restriction enzyme *PvuII*. When cut to completion, *letI*⁺ should be present within a 4.6kb *PvuII* fragment (Michael *et al.*, 1994). The gel is shown in Figure 5.3 (A). The full length 1.9kb *ClaI* fragment from pKO45, containing the entire genomic *letI*⁺

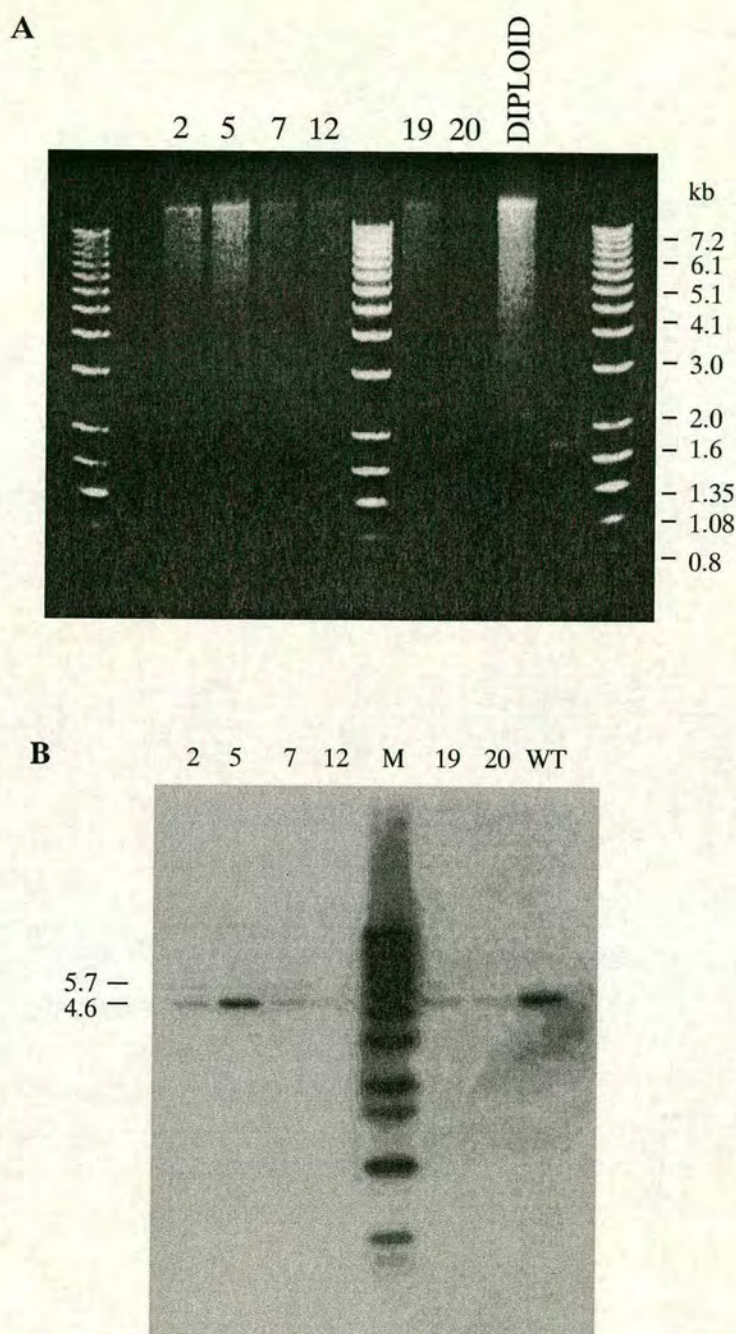


Figure 5.3 Southern blot of genomic DNA from diploid cells carrying a disruption of *let1*⁺.

(A) 20µg of genomic DNA from diploid cells carrying a putative disruption of the *let1* gene was digested with an excess of *PvuII* and run on a 1.2% TAE gel, and stained with ethidium bromide. (B) DNA was transferred to Hybond-N⁺, fixed and probed with a ³²P-labelled probe complementary to the genomic *let1*⁺ gene. Two bands, indicating the presence of a wild-type (4.6kb) and disrupted (5.7kb) copy of the *let1* gene are visible in the lanes labelled 2, 9 and 19. DNA from a wild-type diploid strain was used as a control.

fragment (Michael *et al.*, 1994), was labelled by random priming (see Materials and Methods) and used to probe the *PvuII* digested genomic DNA. Figure 5.3 (B) shows that of the 6 isolates which gave the correct size of PCR product, at least 3 (numbers 2, 9 and 19) gave the 2 bands expected if the *ura4⁺* marker had integrated at the *let1⁺* chromosomal locus.

5.5 Genetic analysis of the disrupted allele

Having deleted a large amount of the *let1⁺* coding region, tetrad analysis was used to investigate whether or not *let1⁺* was essential for cell viability. Following nutrient starvation, a diploid cell enters pre-meiotic S-phase followed by two meiotic divisions to form an ascus of four haploid spores (Bresch *et al.*, 1968). In the *let1Δ::ura4⁺* strain, two of these spores should be wild type at the *let1⁺* locus, but *ura⁻* whereas the other two should carry the *ura4⁺* gene at this position, thus providing a means of selection for viability.

5.5.1 Tetrad analysis of the *let1⁺/let1Δ::ura4⁺* diploid strain

After two days of nitrogen starvation, in order to induce sporulation (Leupold, 1958), cells were checked for their ability to sporulate (see Materials and Methods). No sporulation had taken place indicating that the heterozygous diploids carrying the disrupted *let1* allele had reverted from the heterozygous mating type *h⁺/h⁻* to a homozygous mating type of either *h⁺/h⁺* or *h⁻/h⁻*. This occurs at a frequency of 1×10^{-5} and is a result of mitotic recombination between the mating type loci of the paired chromosomes present in the diploid cell (Beach and Klar, 1984). For further analysis it was necessary to find *h⁺/h⁹⁰* revertants which would then sporulate.

The mating type of the non-sporulating heterozygous diploids was tested by crossing them to haploids of a known mating type and then staining with iodine. Three (2, 19 and 20) which were found to be *h⁺/h⁺* were then used to create sporulating *h⁺/h⁹⁰* re-

vertants (see Materials and Methods). Eighteen asci were pulled from each of the 3 sporulating heterozygous diploids.

Of the tetrads dissected from diploid #2, 14 segregated in a 2:2 viable: non-viable manner (Figure 5.4). The remaining 4 tetrads were not analysed further because most of the spores failed to germinate. The viable spores were patched onto complete media (YPD), minimal media with uracil, adenine and leucine (EMM+U+A+L) and minimal media lacking uracil (EMM+A+L) (Figure 5.5). None of the viable spores were able to grow on media lacking uracil, indicating that they did not carry the uracil marker (Figure 4.5). This suggested that only those spores which had an intact copy of *let1*⁺ were viable and, consequently, that *let1*⁺ was essential for the viability of the cell.

The non-viable spores did germinate and divided either once or twice to give 2 or 4 cells respectively (data not shown). This is possible because there may have been a small amount of *let1*⁺ encoded protein within the cell from the heterozygous diploid, enabling cell cycle progression to occur. However, as the cell continued to divide, the concentration of this protein would decrease thus preventing further cell division.

This experiment showed that *let1*⁺ is essential for cell viability. This situation is identical to disruptions in all but one of the 19S subunits so far studied (van Nocker *et al.*, 1996). Further analysis was required to extend these results and thereby highlight further similarities or differences between the phenotype of a disruption of *let1*⁺ and that of other regulatory subunits of the 26S proteasome. In the case of the *mts2*⁺ disruption, fluorescence activated cell sorter (FACS) analysis revealed that the arrest point during the cell cycle took place after DNA replication. Confirmation of this was obtained by cytological analysis of the cells. The increase in the number (48%) of cells which arrested with a short mitotic spindle and condensed chromosomes showed that this disruption affected the ability of the cell to complete mitosis following DNA replication. These experiments were repeated in order to investigate the phenotype of the *let1* disruption

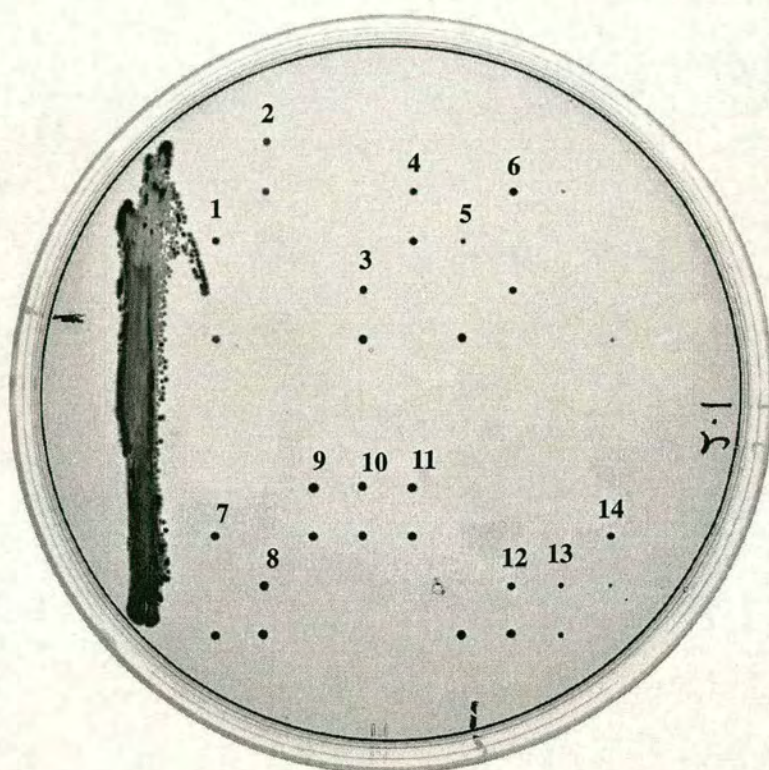


Figure 5.4 Tetrad analysis of a *let1* disruption

Diploid cells carrying a disruption of the *let1* ORF were induced to sporulate on ME media. 18 asci were picked onto YE, incubated at 36°C for 3-4 hours to facilitate breakdown of the ascus wall, then the spores were pulled into rows of 4 and incubated at 25°C. Of these 18 asci, 14 (1-14) produced 2 viable spores, 3 produced only 1 visible colony, and 1 failed to germinate.

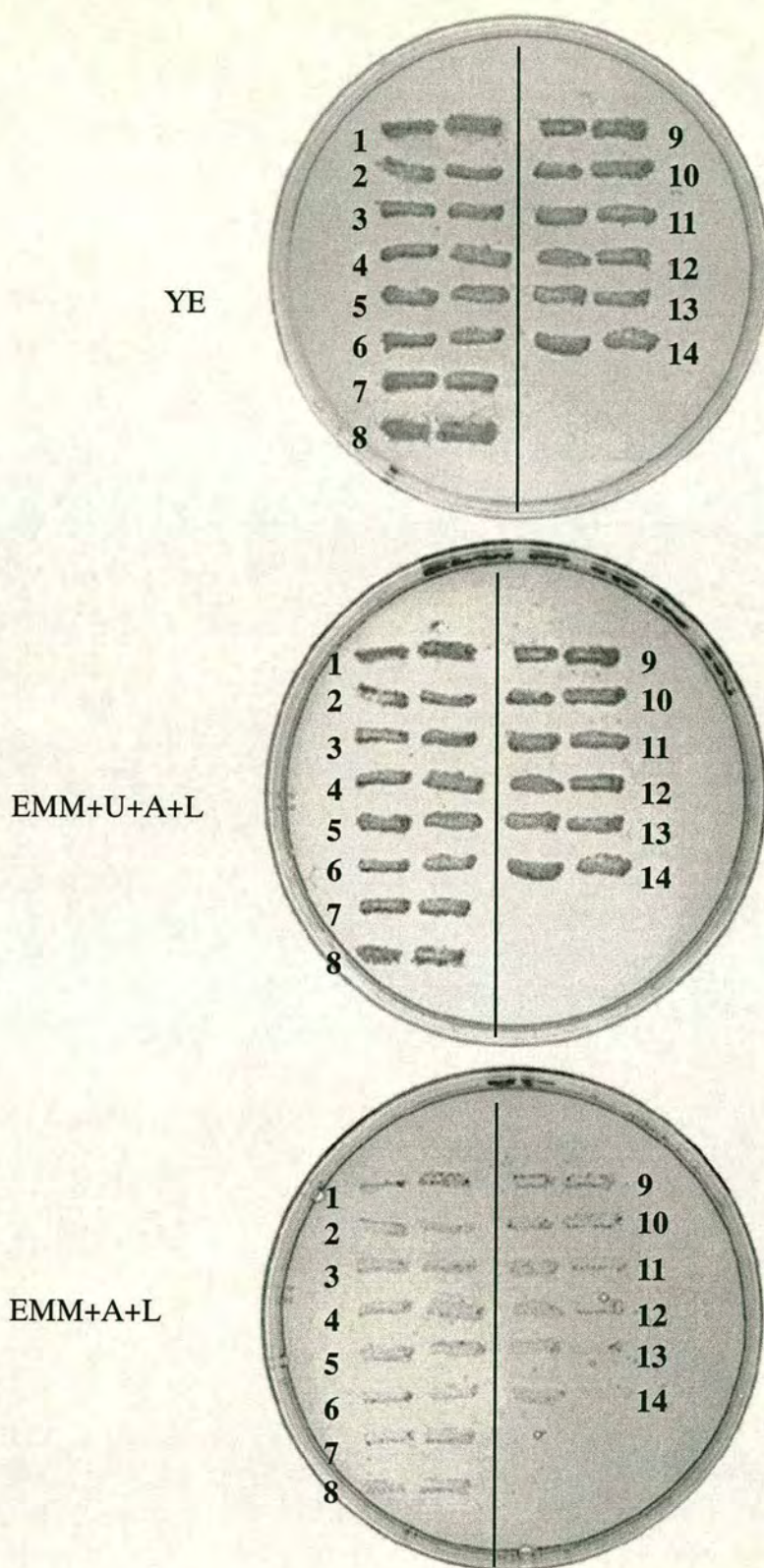


Figure 5.5 Auxotrophy of viable spores from *let1* disruption

Colonies produced from tetrad analysis of the *let1* disruption (see Figure 5.4) were tested for the presence or absence of the *ura4⁺* gene. Viable colonies from asci 1-14 were patched onto YE media (A), EMM+U+A+L (B) and EMM+A+L (C) and incubated at 25°C. None of the patches grew on media without uracil.

5.6 FACS analysis of a *let1* Δ

Measuring the DNA content of a cell by FACS analysis gives an indication of which stage of the cell cycle the cell is at. The fluorescence is based on the excitation of the fluorogenic compound, propidium iodide (PI), which is used to stain the DNA. Photon emission from this compound, following excitation by a laser, is registered as peak and can be graphically displayed. The position of the peak is a direct measure of the amount of DNA within the cell.

Exponentially growing *S.pombe* haploid cells have a predominantly 2n peak whereas the DNA content of ascospores prior to germination will be 1n indicating that only one copy of each of the three *S.pombe* chromosomes is present within the cell. Upon germination however, the DNA replicates thereby increasing the DNA content to 2n. In a wild-type population of spores, all of the spores should have a 1n content of DNA. Following DNA replication, the position of the peak should move to, and remain at, a position corresponding to a 2n amount of DNA.

Analysis of *S.pombe* mutants carrying conditional mutations in genes encoding other 26S proteasome subunits, such as *mts2-1* (Gordon *et al.*, 1993) and *mts4-1* (Wilkinson *et al.*, 1997), has shown that in these cells, the 26S complex acts at a point following DNA replication but before the anaphase stage of mitosis. Consequently, FACS analysis of these mutants, grown at a restrictive temperature, reveals that they arrest with a 2n DNA content. Disruption of the *mts2*⁺ and *mts4*⁺ genes resulted in phenotypes which were similar both to each other and to the corresponding *ts* mutations. This indicated that both of these genes are essential for cell viability and that in both cases, the 26S complex was required at a stage following DNA replication.

The comparison of FACS analysis of spores prepared from an *mts2* Δ ::*ura4*⁺ heterozygous diploid with those prepared from the *let1* Δ ::*ura4*⁺ heterozygous diploid is shown (Figure 5.6). In both cases a large, single peak, corresponding to ascospores

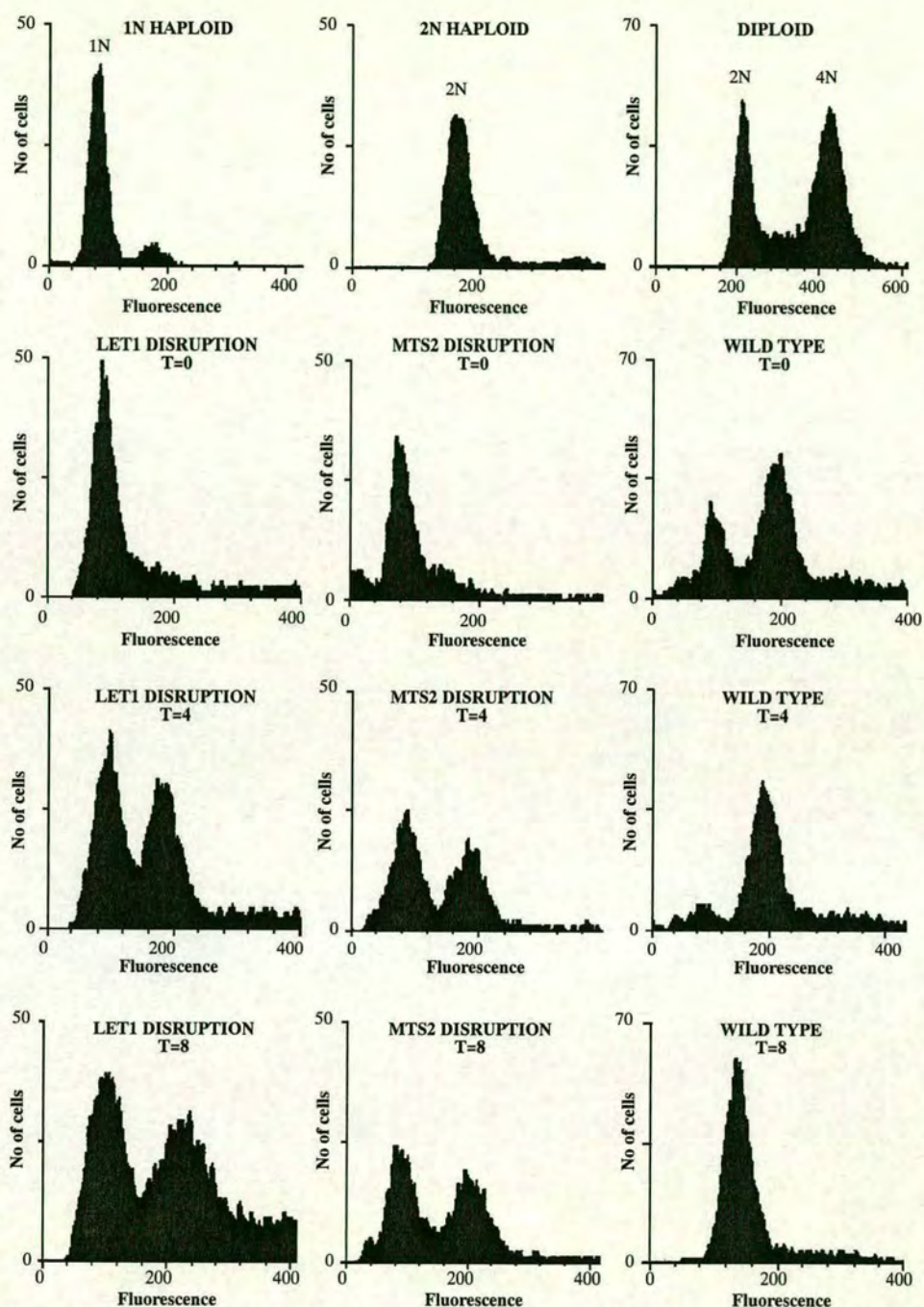


Figure 5.6 FACS analysis of a *let1::ura4+* disruption.

Approximately 10^8 purified spores, from a diploid strain carrying a disruption of the *let1* gene, were germinated for 16 hours at 16°C in 100ml EMM+L, then shifted to 30°C . 5ml samples were taken at 0, 4 and 8 hours, fixed in 70% ethanol, stained with propidium iodide and analysed using a Becton Dickinson FACS machine. The fluorescence of the cells (X-axis), corresponding to the DNA content of the cells, was measured. The position of peaks, corresponding to cells containing 1N, 2N and 4N amounts of DNA, is shown (top row). The Y-axis is the proportion of cells containing that amount of DNA. Cells carrying a disruption of *mts2*, and wild type diploid cells, were used as controls.

containing unreplicated DNA (see control), is initially visible. Following incubation under conditions of nutrient limitation, a second peak appears. For the *let1* disruption, this second (2n) peak corresponds to the spores which carry the *let1* disruption and which have undergone DNA replication.

The 1n peak corresponds to those spores which are not able to replicate due to nutrient limitation, and which therefore do not carry a copy of the *ura4⁺* gene. These spores are wild type at the *let1⁺* locus. This was shown by incubating spores in media containing uracil, whereupon the 1n peak disappeared (data not shown). For disruptions of both *mts2⁺* and *let1⁺*, the relative levels of these 2 peaks does not decrease even after 8 hours, indicating that the arrest point caused by a lack of *let1⁺* or *mts2⁺* function occurs after DNA replication. The 2n arrest point of the spores carrying the *let1* disruption is consistent with the DNA having replicated, and concurs with the results obtained for disruptions of *mts2⁺* and *mts4⁺*.

5.7 Cytological analysis of *let1Δ::ura4⁺*

FACS analysis showed that the phenotypic consequence of disrupting the *let1⁺* gene is very similar to that observed for disruptions in genes encoding other regulatory subunits of the 26S proteasome. If Let1p is a subunit of this complex and not, as previously thought for the *S.cerevisiae* homologue Sug1p, a transcription factor or transcriptional regulator (Swaffield *et al.*, 1992, Kim *et al.*, 1994, Xu *et al.*, 1995), then cytological analysis of cells carrying a disruption of the *let1⁺* gene should highlight a similarity between this disruption and that of *mts2⁺*.

Previously, disruptions of the *mts2⁺* and *mts4⁺* genes had been shown to result in a mitotic arrest. The severity of the arrest was quantitated by observing the number of cells which had a short mitotic spindle and condensed chromosomes, indicative of the metaphase stage of mitosis.

Fluorescence microscopy can be used to visualise cell cycle-associated structures such as the mitotic spindle, the state of chromosome condensation and the presence

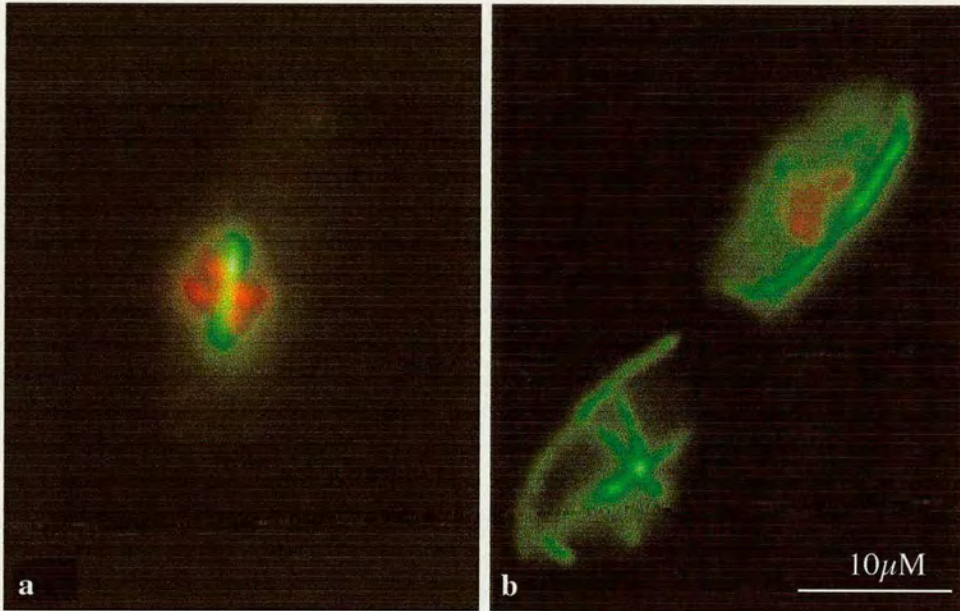


Figure 5.7 Immunofluorescence microscopy of a cell carrying a disruption of the *let1* gene.

S. pombe cells carrying a disruption of the *let1* ORF were fixed in 7% paraformaldehyde, and stained with DAPI (red) and Tat anti-tubulin antibodies (a gift from Iain Hagan). Cells were visualised on a Zeiss fluorescence microscope (x100 magnification). **(a)** After 4 hours incubation at 30°C, cells arrest at metaphase with short mitotic spindle (green) and condensed chromosomes (red). **(b)** After 12 hours, arrested cells continue cell cycle events, giving rise to invaginated cells with one nucleus and a post-mitotic microtubule array.

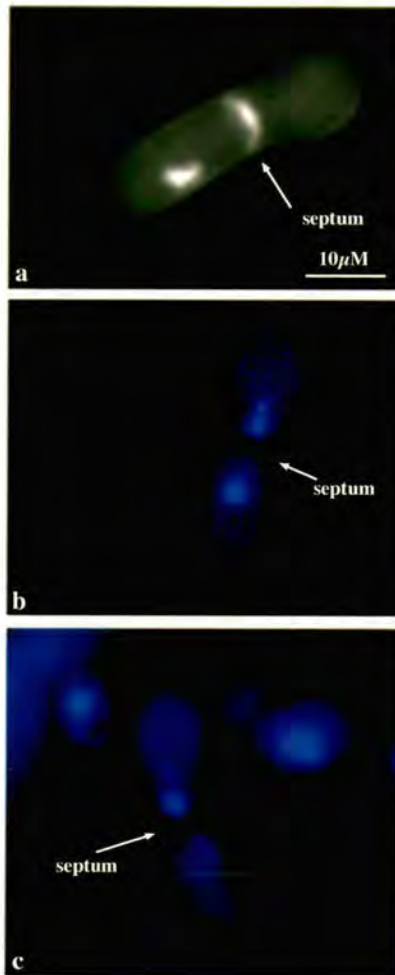


Figure 5.8 DAPI and aniline blue staining of cells carrying a disruption of the *let1* gene.

Sporulating *let1Δ::ura4⁺* cells were sampled after 8 hours of incubation at 30°C in EMMM+A+L, stained with DAPI and aniline blue at a final concentration of 2.5 μg ml⁻¹ and viewed using a Zeiss AXioplan fluorescence microscope at x100 magnification.

a) Aniline blue staining showing position of septum.

b) and c) DAPI staining to show the 'cut' phenotype (Hirano *et al.*, 1986). Failure to segregate chromosomes properly results in the formation of a septum through the chromosomal material. The position of the septa is shown.

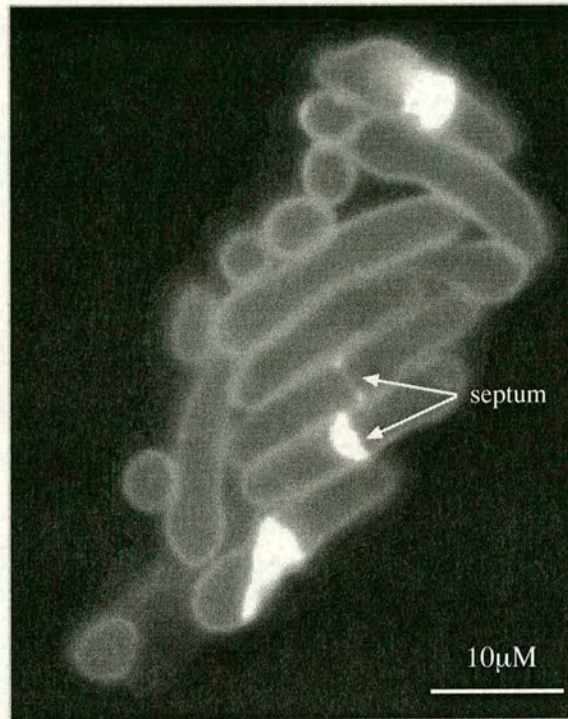


Figure 5.9 Aniline blue staining of sporulating *let1::ura4⁺* cells to show the presence of septa.

Cells were sampled and treated as in Figure 5.8. Approximately 50% of cells carrying a disruption of the *let1* gene form a septum (and see Figure 5.10). Cells carrying this disruption are characteristically elongated and are rounded at one end. Position of the septum is shown.

or absence of septa. These can all be studied by the use of fluorescent labels specific for different components of the mitotic apparatus. Anti-tubulin antibodies (Tat1) were used to label the mitotic spindle. DAPI (4',6-diamidino-2-phenylindole) was used to label chromosomes. Unlike calcofluor, which is commonly used, aniline blue was used to stain septa. This has the advantage that it is specific for septal material (Kippert *et al.*, 1995).

Spores were germinated as before (section 5.5.1) and fixed using paraformaldehyde (see Materials and Methods). The cells were then stained and examined by fluorescence microscopy. The results of these experiments are shown (Figures 5.7-5.9). Figure 5.7 shows the double staining of cells arrested at the metaphase to anaphase transition and the resultant phenotype following subsequent incubation of these cells at 30°C. The mitotic arrest (Figure 5.7 A) is transient, and eventually gives rise to septated cells with only one nucleus (Figure 5.7B). Figure 5.8 (a-c) shows cells which have been stained with aniline blue. In 10-15% of cells a 'cut' phenotype, where the septum has formed through the improperly segregated DNA, is visible (Figure 5.8b). Cells stained with aniline blue (Figure 5.9) show that 50% of the cells arrested at metaphase go on to form septa. This indicates that although the product of the *let1*⁺ gene is required for the metaphase to anaphase transition, it does not seem to be required for septum formation. The relationship between chromatin condensation, septum formation and the number of nucleate / anucleate cells is shown in Figure 5.10.

5.8 Discussion

Tetrad analysis of the heterozygous *let1*⁺/*let1::ura4*⁺ diploid showed that *let1*⁺ is essential for cell viability. This was expected since a spontaneous deletion of the mating locus, which contains the *let1*⁺ gene, is also lethal (Michael *et al.*, 1994). Germinated spores arrested at the metaphase stage of mitosis. 30% of all of the germinated spores underwent septation giving rise to 2 cells with only one nucleus. Frequently this resulted in the 'cut' phenotype in which cytokinesis occurs without proper chromosome segregation (Hirano *et al.*, 1986).

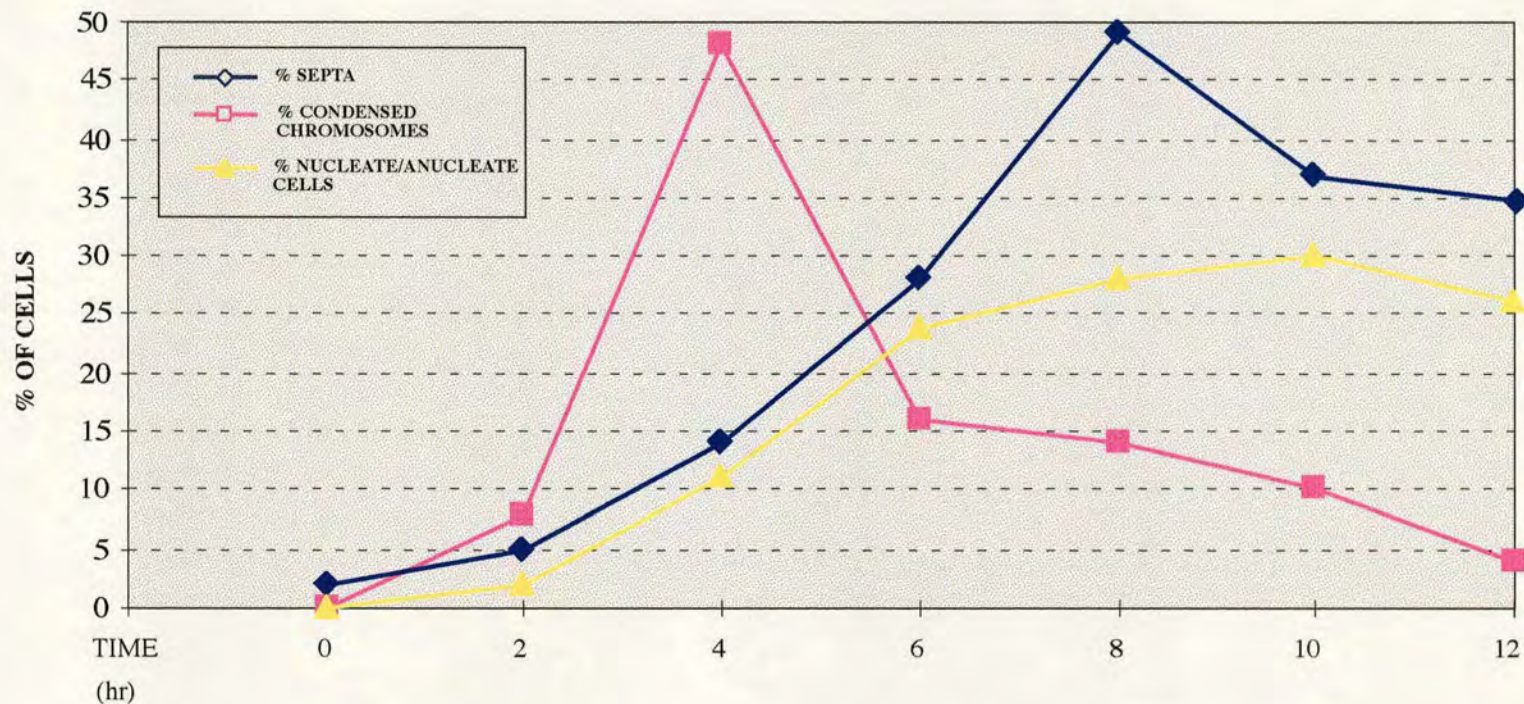


Figure 5.10 Relationship between chromosome condensation, septum formation and time in cells carrying a disruption of the *let1* gene.

Purified spores were germinated in EMM+A+L at 16°C then shifted to 30°C at Time=0. 10^6 spores were harvested at 2 hourly intervals, fixed and stained as described, then examined for the presence or absence of septa, condensed chromosomes and terminal phenotype. 100 cells were counted at each time point. The relationship between these parameters was plotted using Microsoft Excel.

Haploid spores which carried the disruption underwent germination, but were only able to divide once or twice. FACS analysis of these spores confirmed that prior to reaching their terminal phenotype, they underwent one round of DNA replication. Whereas this was similar to observations made for disruptions of the *mts2*⁺ and *mts4*⁺ genes (Gordon *et al.*, 1993; Wilkinson *et al.*, 1997), a disruption of the *mts3*⁺ gene, which also encodes a subunit of the 19S regulatory complex, resulted in a different phenotype (Gordon *et al.*, 1996). In this case, cells undergo two rounds of replication without mitosis, indicating an additional function for the product of the *mts3*⁺ gene in the control of DNA replication. This distinct phenotype may reflect the interaction of Mts3p with positive regulators of S-phase such as Cdc18p, which is stabilised in an *mts3* mutant (Kelly *et al.*, 1993).

Cytological analysis revealed that the *let1* disruption resulted in a large increase in the number of cells which contained a short mitotic spindle and condensed chromosomes. This phenotype is similar to that found in strains carrying null alleles of other 26S proteasome subunit-encoding genes. The main difference between the phenotype of these null alleles is the degree of metaphase arrest which is observed. For the *mts2* disruption, approximately 30% of the cells arrest with a short metaphase spindle and condensed DNA (C. Gordon, unpublished results). This figure was higher (45-50%) when the effect of a disruption of *mts4*⁺ was analysed (Wilkinson *et al.*, 1997). In the present study, a disruption of *let1*⁺ results in a peak (48%) of chromosome condensation which occurred approximately 4 hours after incubation at 36°C. By contrast, the level of septation peaked between 2 and 3 hours later.

The amino acid sequence of Let1p bears 73% similarity to other proteasomal members of the AAA protein family, such as Mts2p, MSS1 and TBP-1 (Gordon *et al.*, 1993; Ghislain *et al.*, 1993; Dubiel *et al.*, 1993 and see Figure 1.3). Therefore, despite its original isolation as a suppressor of a deletion in the *S.cerevisiae* Gal4 protein (Swaffield *et al.*, 1992), it seemed likely that Let1p might also be a subunit of the 26S proteasome complex. The phenotype of cells containing a *let1* null allele supports this theory. This has also been recently confirmed by the immunoprecipitation of Sug1p/ Let1p from purified 26S proteasome extracts from budding yeast (Rubin *et al.*, 1996) and from fission yeast (W. Dubiel, unpublished results, and Figure 5.11).

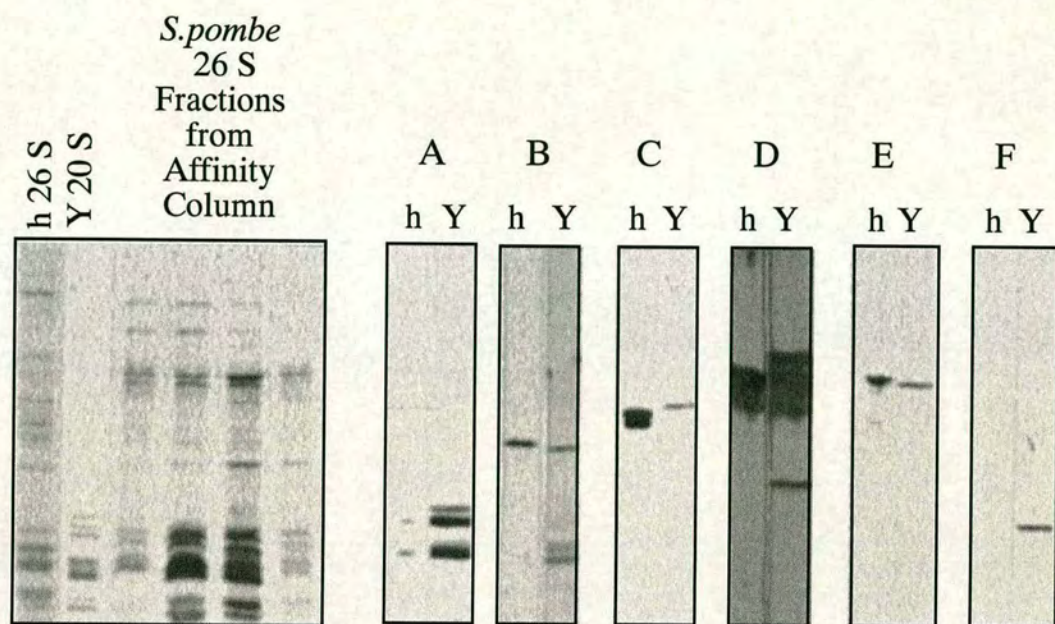


Figure 5.11 Let1p is present in the *S.pombe* 26S proteasome
(Wolfgang Dubiel, unpublished results)

Purified 26S proteasome (10µg) fractions from human (h) and *S.pombe* (Y) cells were blotted onto nitro-cellulose and probed with antibodies raised against subunits of the 26S proteasome. Panels (A) anti-20S proteasome (B) anti-Cim3p (Sug1p) (C) anti-Cim5p(Mss1p) (D) anti-Mts1p (E) anti-Mts2p (F) anti-Mts3p

In this latter case, antibodies raised against the budding yeast homologue of Let1p (Cim3/ Sug1) were used. These latter results demonstrated that in contrast to previous reports, an *S.pombe* homologue of Cim3 was present in the 26S proteasome. The homologue of Cim5 was also present.

Chapter 6

Chapter 6 Characterisation of mutations in the *mts2* gene.

6.1 Introduction

The introduction of nonsense mutations into the ORF of a gene result in the premature truncation of the gene product. As seen in Chapter 4, expression of C-terminally truncated forms of Let1p, produced by PCR-generated deletions in the *let1*⁺ gene, cause a dominant negative phenotype (Herskowitz, 1987). Given the evidence presented so far, it seems that this may happen because these truncations can still bind neighbouring subunits of the 26S proteasome, thereby precluding its proper formation.

Nonsense mutations can also give rise to a conditional phenotype. The *mts1*⁺ gene of *S.pombe* encodes a non-ATPase subunit of the 26S proteasome (C. Gordon, unpublished result). Mutations in this gene are predicted to result in the premature truncation of the *mts1* gene product (P. Kersey, unpublished results). Just as for other mutants isolated in the same screen, all of the *mts1* alleles are *ts*. Experiments are being done to ascertain whether the truncation of the *mts1-1* gene product precludes its assembly into the 26S complex at the restrictive temperature.

By contrast, point mutations which cause an alteration in the primary structure of a polypeptide can also give rise to a conditional phenotype which can be exacerbated by subjecting the protein to environmental stresses such as extremes of temperature. The severity of the phenotype which occurs as a result of these conditional lethal mutations is most likely dependent on the specific alteration in the quaternary structure of the mutated polypeptide.

Without knowing the crystal structure of a protein, it is difficult to predict exactly how amino acid changes contribute to the lability of the proteins. It can be imagined that if an uncharged residue is replaced in a mutated polypeptide by a charged resi-

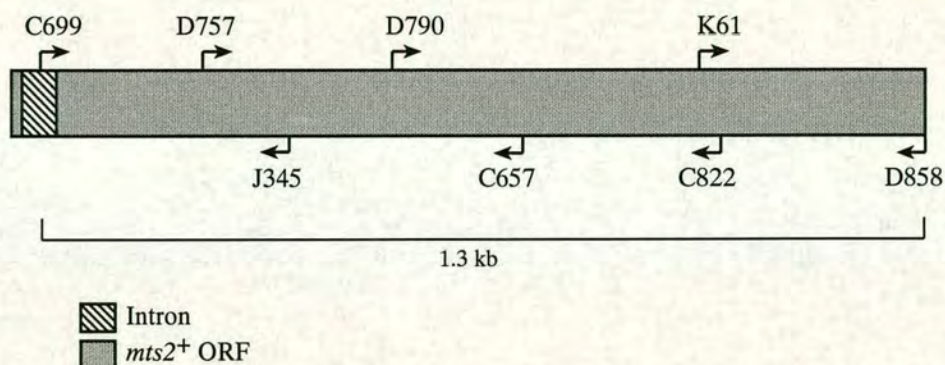
due, the resultant net charge difference may affect the folding of the protein or the association between individual residues. In the same way, the substitution of a small residue, such as glycine, by a large residue, such as phenylalanine, may impose a steric effect resulting in an unstable ternary structure. If these mutations occur in a conserved 'domain' or binding-motif of a protein, they may affect the function of that domain. The analysis of conditional lethal mutations, therefore, can give us some clues as to how different amino acid substitutions result in different phenotypes.

The genetic screen to isolate MBC^R *ts* mutants led to the isolation of genes which encoded subunits of both the regulatory and catalytic complexes of the 26S proteasome (Gordon *et al.*, 1993, 1996 and unpublished results). Subsequent analysis of mutants isolated in this screen has led to the discovery of 2 more alleles of *mts2*: *mts2-16* and *mts2-25*. When these two alleles were crossed to the *mts2-1* mutant and the phenotype of the meiotic products was analysed, all of the spores analysed were *ts*, indicating that no recombination had occurred. The mutations were therefore tightly linked. Also, when transformed with the *mts2*⁺ cDNA, the conditional phenotype of these mutants was rescued.

In a separate experiment to study mutants which affected centromeric repression, a strain of *S.pombe*, carrying the *ura4*⁺ marker integrated at a centromere, was subjected to EMS mutagenesis. Eight *cs* mutants, that expressed the *ura4*⁺ gene, were isolated (J-P. Javerzat, pers. comm.). These mutants are termed *cep* (*cs* enhancement of position-effect variegation) and belong to 3 complementation groups, *cep1-3*. When *cep2* was transformed with a genomic library, a plasmid, containing a 5kb insert, was cloned which rescued the *cs* phenotype of *cep2*. This DNA was shown to contain the entire *mts2*⁺ ORF (Javerzat *et al.*, manuscript in preparation). In total, 4 alleles of *cep2* were isolated. The analysis of these alleles, *cep2-10*, *2-11*, *2-12* and *2-13* in addition to the *mts2* alleles *2-1*, *2-16* and *2-25*, is described here.

6.2 Genetic isolation of *mts2-16* and *mts2-25* alleles

- A**
1. Pick 2 colonies from each mutant for PCR.
 2. Perform PCR reaction using oligonucleotides C699 and D858.
 3. Clean PCR product and sequence both strands.
 4. Run samples on ABI sequencer.
 5. Compare sequence with *mts2*⁺ sequence and find mutation(s).



Oligonucleotide used	Position on <i>mts2</i> ⁺ gene
C699	In intron after A of ATG
D757	243 - 262
D790	613 - 633
K61	938 - 957
D858	1297 - 1279
C822	1137 - 1127
C657	711 - 690
J345	334 - 311

B

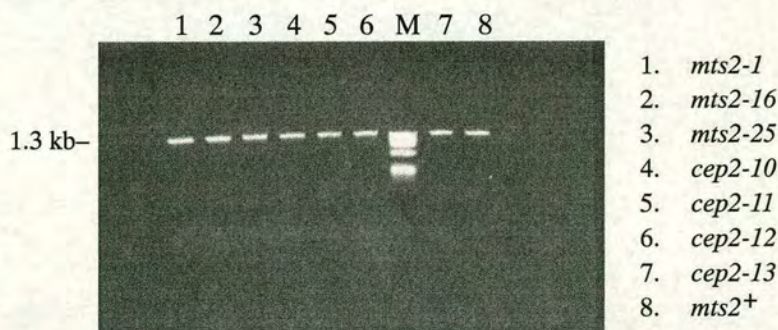


Figure 6.1 Genomic PCR and sequencing of mutations in the *mts2* gene from *mts2* and *cep2* mutants.

(A) Strategy used for sequencing of mutant alleles. The positions of oligonucleotide primers relative to the *mts2* ORF are shown. The sequences of all oligonucleotides used in this amplification procedure are detailed in Appendix A. (B) Genomic DNA was purified from cultures of all mutant strains. Approximately 100ng of genomic DNA was amplified using the primers C699 and D858. Of a 100μl reaction, 5μl was run on a 0.8% TAE gel. Contents of each lane are shown.

UV mutagenesis can be used to induce random mutations in DNA. Typically this occurs because covalent bonds are introduced between two adjacent pyrimidine bases, resulting in the formation of pyrimidine dimers. Thymine dimers are the most frequent type of lesion associated with UV treatment. Cytosine dimers or mixed thymine-cytosine dimers are also produced by UV treatment. The frequency at which any of these forms occurs is dependent on the base composition of the DNA (Fincham, 1983).

During UV irradiation, mutations accumulate at a roughly linear rate. The number of 'hits' can be approximately controlled by varying the dose of radiation applied to the sample. It is possible that the phenotype which results from the UV mutagenesis procedure may be due to the action of more than one 'hit'. Therefore, before doing any analysis on these or other alleles, any unlinked mutations had to be crossed away from the *mts2* gene. This had already been done for alleles of the *cep2* mutant, which were generated by ethylmethane sulphonate (EMS) (J-P. Javerzat, pers. comm.) but remained to be done for *mts2-16* and *mts2-25*.

Fresh *h⁻* isolates of *mts2-16* and *mts2-25*, which had been transformed with the *mts2⁺* cDNA in order to improve mating efficiency, were crossed to a wild-type isolate of the opposite mating type. These plasmids were later lost during meiosis because they don't possess a centromere sequence. Eighteen tetrads were dissected from each cross. The colonies which grew from the resulting spores were patched and tested for MBC^{R} , temperature sensitivity and mating type. As expected, MBC^{R} and temperature sensitivity co-segregated with a 2:2 ratio of $\text{MBC}^{\text{R}}/\text{ts}^- : \text{MBC}^{\text{S}}/\text{ts}^+$. This process was repeated twice so that the isolates of *mts2-16* and *mts2-25* used for subsequent analyses were isogenic.

6.3 Sequence analysis of the *mts2* gene from MBC^{R} and *cep2* alleles

The mutation in the *mts2-1* mutant involved a nucleotide change at position +857. This C-T transition results in the change from a serine to a phenylalanine residue at

position 286 in the protein, 5 amino acids upstream of the DEID box motif, associated with Mg^{2+} binding (L. Colleaux, unpublished result, and see Figures 6.3 and 6.4). This 'Walker B' motif (Walker *et al.*, 1982) lies within the CAD region of Mts2p and is highly conserved in all members of the AAA family of proteins (Confalonieri and Duguet, 1995).

The strategy for sequencing the genomic sequence of the *mts2*⁺ gene is shown in Figure 6.1. DNA containing all but the C-terminal 40 nucleotides was amplified from genomic DNA of each of the putative *mts2* mutants using the oligonucleotides shown. In order to obviate the problem of contamination by *mts2*⁺ cDNA, the oligonucleotide C699, which maps to the intron within the *mts2*⁺ genomic fragment, was used. The 1.34kb fragments amplified by this procedure are also shown.

The products of the PCR reactions were purified, labelled using an ABI sequencing kit and sequenced. The oligonucleotides used for sequencing were designed to enable complete sequencing of the PCR fragment in both directions. The DNA was sequenced from 2 independent PCR products. This approach obviates the possibility of encountering a mutation caused as a result of the PCR reaction. Throughout all of this sequencing procedure, no PCR-generated mutations were observed. The sequences were analysed using the software provided, and compared to the sequence of *mts2*⁺ by means of the UWGCG software. The output and comparison between mutant and wild-type *mts2* sequences is shown in Figure 6.2A-6.2G. The nature and position of these mutations is shown in Figure 6.3 and Figure 6.4.

The most interesting aspect of these results is the position of the mutations and the allelic nature of two of the *cep2* mutations. In the case of *mts2-25* and *cep2-11* and *cep2-12*, Figures 6.2C, E and F, and 6.3-6.4 show that these mutations occur in adjacent glycine residues within the conserved region of Mts2p. Whereas a change to arginine, at position 195 in the product of *mts2-25*, results in a *ts* phenotype, a change in the adjacent glycine residue at position 194 in the products of *cep2-11* and *cep2-12*, to aspartate, results in a *cs* phenotype. Sequencing of *cep2-11* and *cep2-12* showed that the same mutation, a G to A transition, occurred in each case. Since

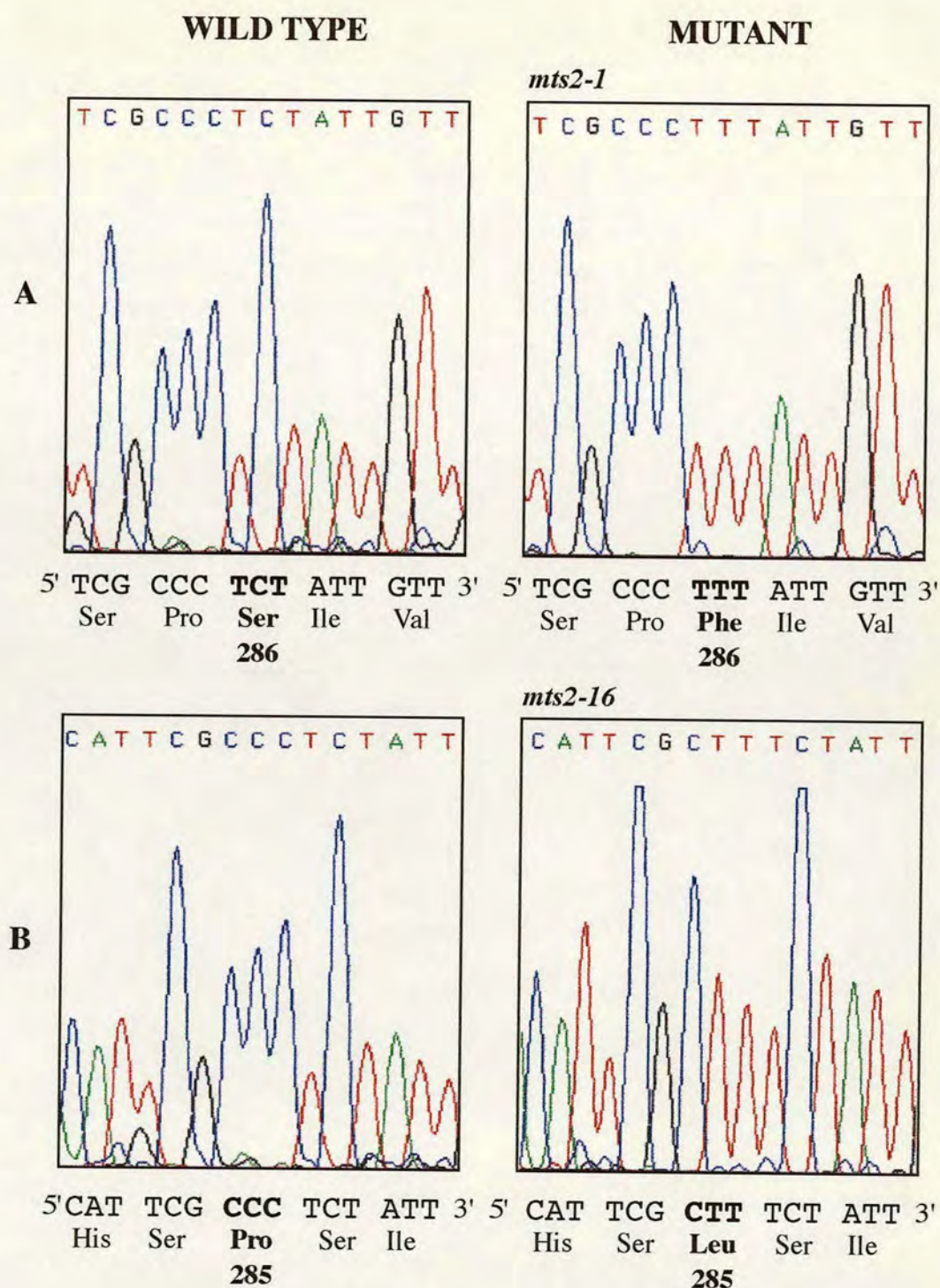


Figure 6.2 DNA sequence of mutations in alleles of *mts2* and *cep2*.

The *mts2* gene from all alleles of *mts2* and *cep2*, as well as from a wild-type *S.pombe* strain, was amplified by PCR as described. The DNA was sequenced in both directions, using the primers shown in Figure 6.1 by means of an automated ABI sequencer. Mutations found within the *mts2* gene were analysed and graphically displayed using ABI 373 software. The corresponding region of the *mts2+* gene from a wild type strain, sequenced in parallel, is also displayed.

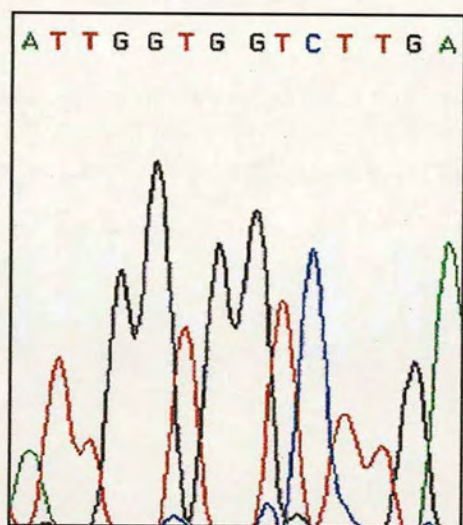
(A) *mts2-1* (B) *mts2-16* (C) *mts2-25* (D) *cep2-10* (E) *cep2-11* (F) *cep2-12* (G) *cep2-13*

WILD TYPE

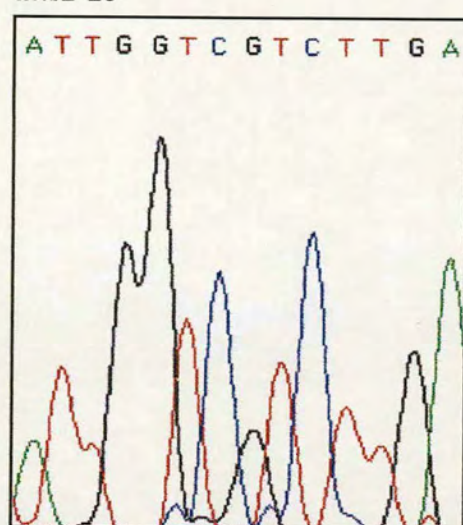
MUTANT

mts2-25

C



Ile Gly **Gly** Leu Glu
195



Ile Gly **Arg** Leu Glu
195

WILD TYPE

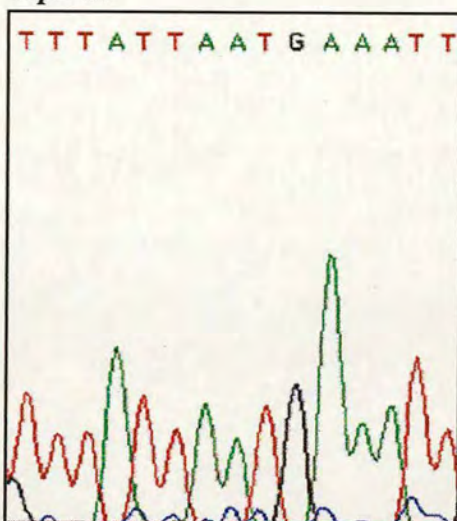
MUTANT

D



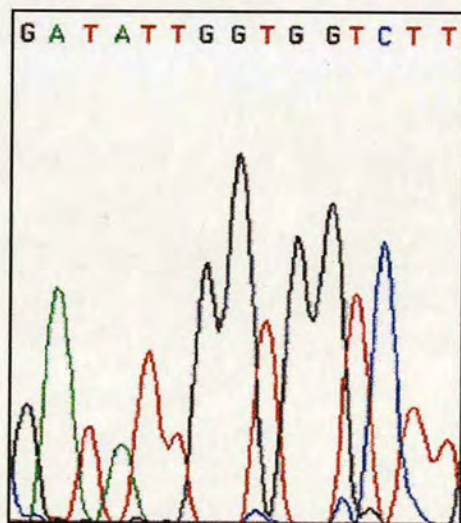
5' TTT ATT **GAT** GAA ATT
Phe Ile **Asp** Gln Ile
291

cep2-10



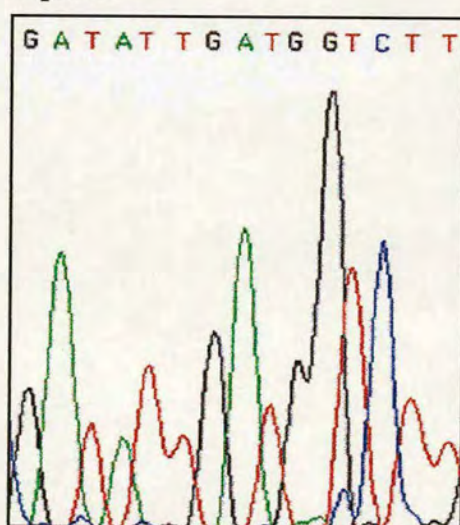
5' TTT ATT **AAT** GAA ATT 3'
Phe Ile **Asn** Gln Ile
291

E



5' GAT ATT **GGT** GGT CTT 3'
Asp Ile **Gly** Gly Leu
194

cep2-11

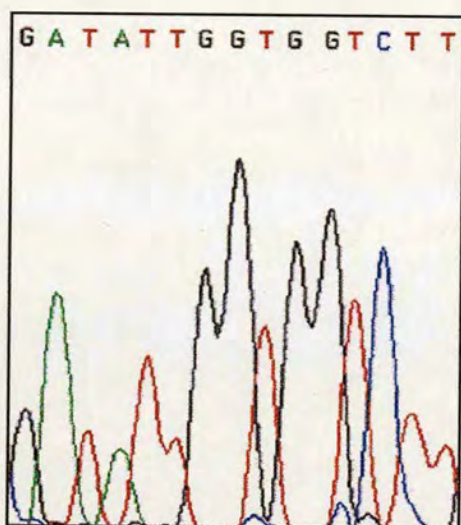


5' GAT ATT **GAT** GGT CTT 3'
Asp Ile **Asp** Gly Leu
194

WILD TYPE

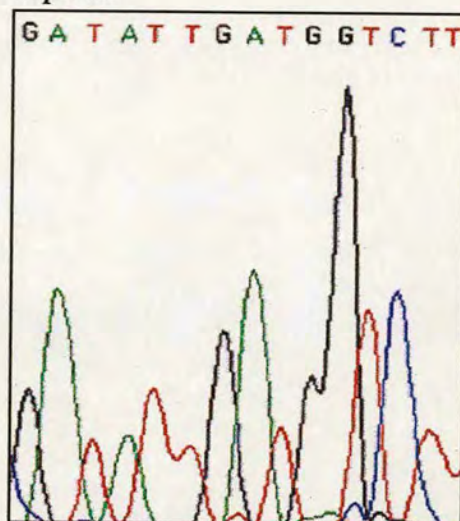
MUTANT

F



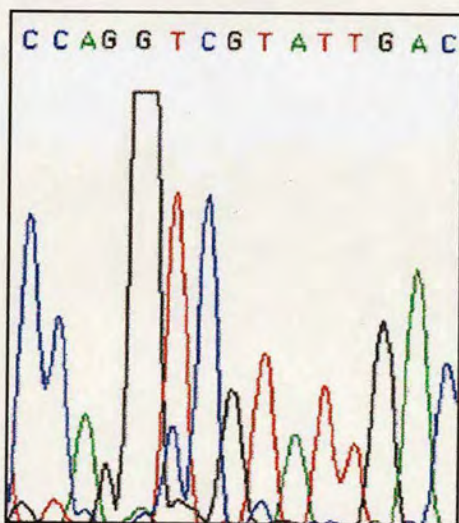
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Asp Ile **Gly** Gly Leu
194

cep2-12



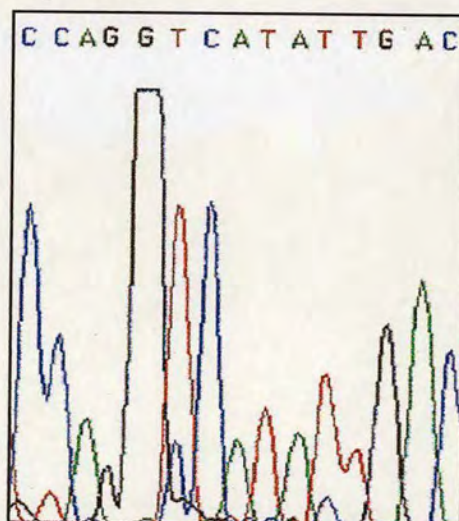
5' GAT ATT **GAT** GGT CTT 3'
Asp Ile **Asp** Gly Leu
194

G



5' CCA GGT **CGT** ATT GAC 3'
Pro Gly **Arg** Ile Asp
353

cep2-13



5' CCA GGT **CAT** ATT GAC 3'
Pro Gly **His** Ile Asp
353

these two mutants were isolated independently of each other, and were therefore not clonal in origin, their allelic nature is quite coincidental.

The mutations in *mts2-1* and *mts2-16* also occur in adjacent codons. In *mts2-1*, the change from serine, a small, polar residue, to phenylalanine, a large aromatic residue, at position 286 occurs as a result of a C-T change at base number 857 (Figure 6.2A). This may be the result of a change to a mixed pyrimidine dimer, as mentioned earlier. In *mts2-16*, a classical pyrimidine dimer transition is responsible for the change from proline to leucine at position 285 of the *mts2-16* gene product (Figure 6.2B).

Perhaps the most interesting mutations are those found in *cep2-10* and *cep2-13*. The mutation in the *cep2-10* allele (Figure 6.2D) results in a change from aspartate, a negatively charged residue, to asparagine, a positively charged residue, at position 291. The aspartate residue at this position is conserved in all members of the AAA family (Confalonieri and Duguet, 1995, and see Figure 1.6) and forms part of the DEID box, a motif thought to be involved in the binding of Mg^{2+} , a co-factor essential for the function of these ATPases. This motif is known to be conserved in the DEAD-box family of RNA helicases (see Figure 7.3), suggesting that the phenotype associated with the *cep2-10* mutation may be due to a failure of this putative function.

The mutation in *cep2-10* may still enable the correct folding of the protein and binding of magnesium. However, the phenotype caused by this mutation may be more severe due to a decreased ability to bind the magnesium which is required as a co-factor during ATP hydrolysis. If this is the case, it is possible that an alteration in intracellular magnesium levels may partially or totally suppress the *cs* phenotype when the mutant is subjected to restrictive conditions.

Like *cep2-10*, the *cep2-13* mutation (Figure 6.2G) lies in a motif conserved in the DEAD box family of RNA helicases (Gorbalenya *et al.*, 1989). The mutation results in a change in the predicted peptide from a highly conserved arginine to histidine (see Figure 6.4 and Figure 7.3). Both of these amino acids carry positive charges which

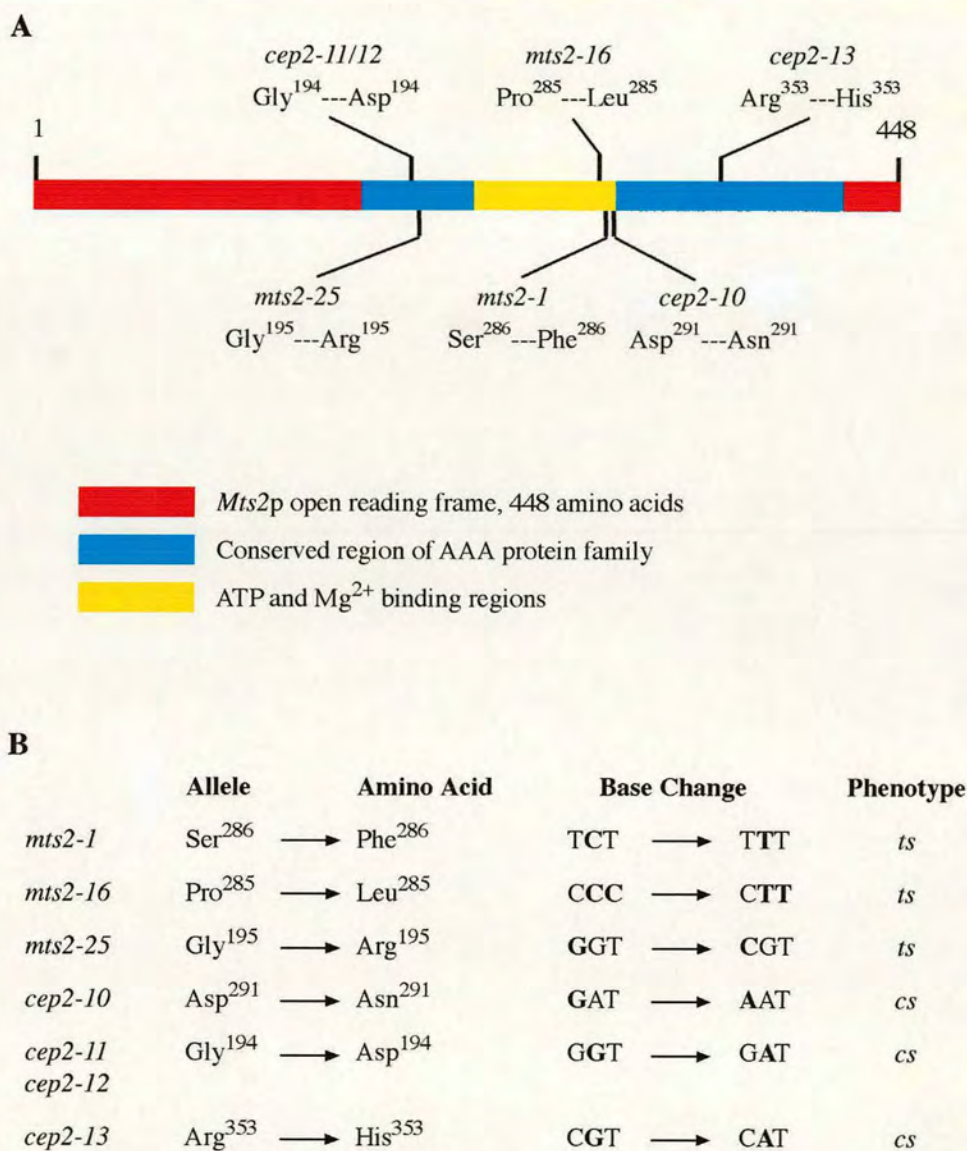


Figure 6.3 Position of mutations in alleles of *mts2*

The position of all sequenced mutations in the *mts2* gene relative to the ATP-binding cassette and the region conserved in AAA protein family members is shown (A) The genotype and conditional phenotype of all mutations is also shown (B)

<i>Mts2</i> ⁺	MGQAQSGNFS	NFGDGANGDN	KKDQKKDKPK	YEPPVPTRTG	RRKKKAQSGP
<i>mts2</i>	MGQAQSGNFS	NFGDGANGDN	KKDQKKDKPK	YEPPVPTRTG	RRKKKAQSGP
<i>Mts2</i> ⁺	DASAKLPTVI	PTTRCRLRL	KMQRIHDHLL	MEEYVQNQE	RLKPQDERTQ
<i>mts2</i>	DASAKLPTVI	PTTRCRLRL	KMQRIHDHLL	MEEYVQNQE	RLKPQDERTQ
<i>Mts2</i> ⁺	EERNRVDEIR	GTPMSVGTLE	EIIDDDHAIV	STAGPEYYVS	IMSFVDKML
<i>mts2</i>	EERNRVDEIR	GTPMSVGTLE	EIIDDDHAIV	STAGPEYYVS	IMSFVDKML
<i>Mts2</i> ⁺	EPGCSVLLHH	KAMSIVGLLL	DDTDPMINVM	KLDKAPTESY	ADIGGLESQI
<i>mts2</i>	EPGCSVLLHH	KAMSIVGLLL	DDTDPMINVM	KLDKAPTESY	ADIDRLESQI
				<i>cep2-11/cep2-12</i>	<i>mts2-25</i>
<i>Mts2</i> ⁺	QEIKEAVELP	LTHPELYEEM	GIKPPKGVIL	YGAPGTGKTL	LAKAVANQTS
<i>mts2</i>	QEIKEAVELP	LTHPELYEEM	GIKPPKGVIL	YGAPGTGKTL	LAKAVANQTS
<i>Mts2</i> ⁺	ATFLRVVGSE	LIQKYLGDGP	RLVRQLFNAA	EEHSPSIVFI	DEIDAIGTKR
<i>mts2</i>	ATFLRVVGSE	LIQKYLGDGP	RLVRQLFNAA	EEHSLFIVFI	NEIDAIGTKR
			<i>mts2-16</i>	<i>mts2-1</i>	<i>cep2-10</i>
<i>Mts2</i> ⁺	YDAQSGAERE	IQRTMLELLN	QLDGFDTQR	DIKVIMATNR	ISDLDPALIR
<i>mts2</i>	YDAQSGAERE	IQRTMLELLN	QLDGFDTQR	DIKVIMATNR	ISDLDPALIR
<i>Mts2</i> ⁺	PGRIDRKILF	ENPDEATKRK	IFTIHTSKMN	LGEDVNLEEL	IQCKDDLGA
<i>mts2</i>	PGHIDRKILF	ENPDEATKRK	IFTIHTSKMN	LGEDVNLEEL	IQCKDDLGA
	<i>cep2-13</i>				
<i>Mts2</i> ⁺	EIKAIVSEAG	LLALRERRMR	VVMDDFRQAR	EKVLKTKDEG	GPAGGLYI
<i>mts2</i>	EIKAIVSEAG	LLALRERRMR	VVMDDFRQAR	EKVLKTKDEG	GPAGGLYI

Figure 6.4 Predicted mutations in the peptide sequence of Mts2p.

Comparison of the peptide sequence of the wild-type Mts2p protein (upper sequence) to the predicted sequence of Mts2p containing the mutations present in the *mts2* and *cep2* ORF (lower sequence). The Walker-A and Walker-B motifs are shown in yellow.

may enable the protein encoded by the *cep2-13* mutant to fulfil its role(s) at the permissive temperature.

6.4 Phenotype of *mts2* alleles

The cell-cycle phenotype of strains of *S.pombe* carrying conditional mutations in genes encoding subunits of the 26S proteasome has been documented. The restrictive temperature results in a mitotic arrest, after replication, at the metaphase-anaphase transition. In addition to this, the ubiquitous function of the proteasome results in a pleiotropic phenotype involving cell size, shape and probably many other intracellular processes.

Given the nature and location of the mutations described in the preceding section, it seemed reasonable to assume that the phenotype of all mutants carrying a mutation in the *mts2* gene should be very similar if not identical with respect to FACS analysis, cytology, morphology and drug-resistance.

6.4.1 FACS analysis of *mts2/cep2* mutants

Cultures of *mts2-1*, *mts2-16*, *mts2-25* and wild type cells were grown to an OD₆₀₀ of 0.2 and shifted to 36°C. Samples were taken hourly for FACS analysis and cytological procedures. The result of these experiments are shown in Figure 6.5A. From this, it is evident that after 8 hours at the restrictive temperature, like *mts2-1*, *mts2-16* and *mts2-25* behave in the same manner. That is, the 2n peak is always prevalent suggesting that the potential arrest point occurs after DNA replication. The staining of cells with anti-tubulin antibody (data not shown) confirms that all of these *mts2* alleles arrest with a short metaphase spindle and condensed DNA. This is in accordance with the observations on the *mts2-1* mutant (Gordon *et al.*, 1993).

FACS analysis of the *cep2* alleles (Figure 6.5B) revealed a similar phenotype to that observed for *mts2-1*. Again, as would be expected for a mutation in the *mts2* gene, a sharp 2n peak of replicated DNA was visible after 8 hours of incubation at 18°C.

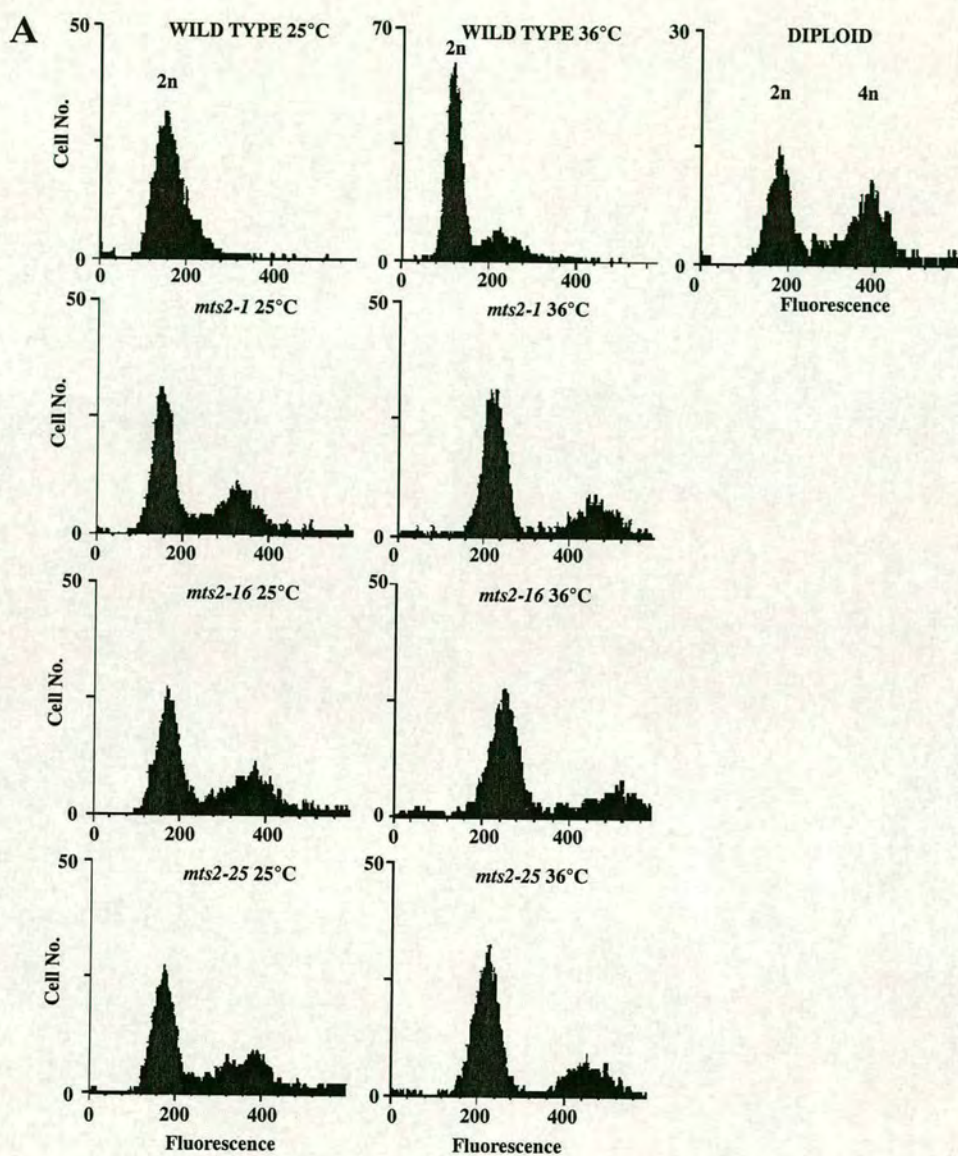
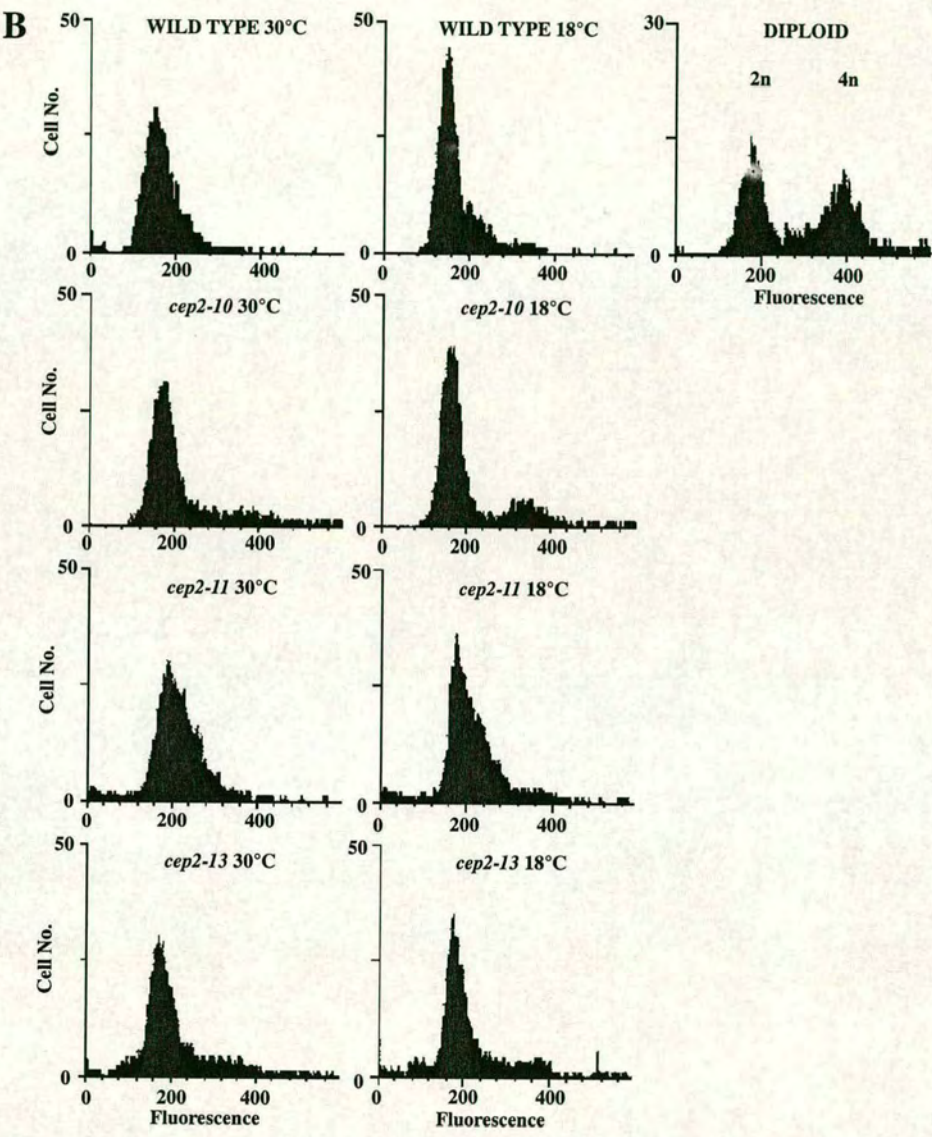


Figure 6.5 FACS analysis of *mts2* and *cep2* mutants

Flow cytometry was performed on asynchronous cultures of cells from all of the strains carrying a mutation in the *mts2* gene. The cultures were shifted from the permissive (25°C for *mts2-1*, *2-16* and *2-25*, 30°C for *cep2-10*, *2-11* and *2-13*) to the restrictive (36°C and 18°C) temperature. Following incubation at the respective restrictive temperatures for 4 hours, 5ml samples were taken, fixed and stained with propidium iodide. (A) FACS analysis of *mts2-1*, *mts2-16* and *mts2-15*. (B) FACS analysis of *cep2-10*, *2-11* and *2-13*. Wild type cells were used as controls in each case. The position of 2N and 4N peaks in a diploid strain are shown.



Despite this similarity, tubulin and DAPI staining highlighted differences in the phenotype of *mts2* and *cep2* mutants. All of the *cep2* alleles display a lagging chromosome phenotype. That is, during anaphase when the segregated sister-chromatids are pulled to opposite ends of the lengthening anaphase spindle (see Figure 1.4), chromosomes were visible along the length of the spindle (J-P. Javerzat, pers. comm.). This phenotype is suggestive either of inefficient binding of microtubules to the centromere or, more likely, of a delay in sister-chromatid segregation. This has not been observed in *mts2* despite *cep2* carrying a mutation in the *mts2* gene. One explanation is that there may be a mutation in another gene which also affects chromosome segregation during mitosis. This is unlikely because any unlinked mutations were crossed away by backcrossing of these *cep2* alleles to a wild-type strain (J-P. Javerzat, pers. comm.). A more plausible explanation is that mutations in the *cep2* alleles of *mts2* affect some other unidentified role of the *mts2*⁺ gene product. As suggested in section 6.3, this role may involve the putative helicase function of Mts2p. A final possibility is that the *cep2* mutations are not as effective in blocking chromosome segregation as those of the *mts2* mutants. This being so, the phenotype which is observed may be due to a failure to degrade Cdc13p, which is also degraded by the 26S proteasome, rather than to a failure to carry out chromosome segregation by degradation of the chromosome 'glue'.

6.4.2 Drug resistance of *mts2* alleles

Multi drug resistance (MDR) in *S.pombe* is conferred by the co-ordinated expression of a number of genes which requires the action of Pap1p, the homologue of the mammalian AP-1 transcription factor (Adachi and Yanagida, 1989). Levels of Pap1p have been shown to be regulated by the transcription factor regulator Pad1p (Shimanuki *et al.*, 1995). Pad1 was recently shown to be a subunit of the 26S proteasome in fission yeast (M.Penney, pers. comm.). Pap1p is thought to be degraded by the 26S proteasome since 26S proteasome mutants, which have defective 26S proteasome function at the restrictive temperature, contain elevated levels of Pap1 transcription factor. The increase in levels of Pap1p coincides with an increase in the transcription of genes essential for MDR (M. Penney, unpublished results).



Figure 6.6 MBC resistance of *mts2-1*, *mts2-16* and *mts2-25*

Fresh isolates from each of the above strains were streaked onto YE media containing increasing 0, 10, 20 and 30g ml⁻¹ of MBC. The plates were incubated at 25°C. Wild type and *mts5-1* strains were used as controls.



Figure 6.7 MBC resistance of *cep2-10*, *cep2-11*, *cep2-12* and *cep2-13*

Fresh isolates from each of the above strains were streaked onto YE media containing 0 and 10g ml⁻¹ of MBC. The plates were incubated at 30°C until colonies appeared. Wild type *S.pombe* and *mts2-1* were used as controls.

A comparison of the 5 original *mts* mutants showed that *mts1-mts4* were equal in their resistance to MBC, whereas *mts5* was approximately five-fold more resistant to this drug. Subsequently, it was discovered that *mts5* contained a mutation in the *crm1*⁺ gene (M. Penney, unpublished result), whereas *mts1*⁺-*4*⁺ were shown to encode subunits of the *S.pombe* 26S proteasome (Gordon *et al.*, 1993, 1996 and unpublished result; C. Wilkinson *et al.*, 1997). The product of *crm1*⁺ has recently been shown to be involved in the export of mRNA from the nucleus (Stade *et al.*, 1997), although mutations in this gene have previously been shown to confer resistance to caffeine and Brefeldin A (Kumada *et al.*, 1996). It is currently unknown how mutations in this gene give rise to an increase in drug-resistance.

The *mts2* and *cep2* alleles were tested for their resistance to MBC. Cells from each allele were streaked onto complete media containing increasing concentrations of MBC and grown at 30°C. Figure 6.6 shows the level of resistance of the alleles *mts2-1*, *2-16* and *2-25* relative to *mts5* and *cdc25* and wild type. Both *mts2-16* and *mts2-25* are slightly more resistant than the *mts2-1* mutant and still show some growth at an MBC concentration of 30 µg ml⁻¹. The *mts5* mutant grows at MBC concentrations of over 50 µg ml⁻¹ (data not shown). Both wild type and *cdc25-22*, which carries a *ts* mutation in a cell-cycle gene, are sensitive to low levels (2-4 µg ml⁻¹) of MBC.

The resistance of the *cep2* alleles is shown in Figure 6.7. In this case, the experiment was performed at a higher temperature of 30°C, due to the *cs* nature of these mutants. All of these alleles are more resistant than wild type, and *cep2-10* is as resistant as *mts2-1* at this temperature. None of the *cep2* mutants was resistant to more than 10 µg ml⁻¹ MBC (data not shown).

The results of these experiments suggest that, like *mts2-1*, *2-16* and *2-25*, the *cep2* alleles are MBC^R to varying degrees, with *cep2-10* being the most MBC^R. Given that all of the *cep2* alleles carry a mutation in the *mts2* gene, this is expected. However, the level of MBC^R may reflect the severity of the phenotype. That is, in the *cep2* mutants, there may be more proteasome function than in the *mts2* mutants. This would account for the observed difference in cytological phenotype with the *mts2* mutants arresting at metaphase, and the *cep2* mutants passing this stage of mitosis.

6.4.3 Morphology of *cep2* and *mts2* alleles

Another means of analysing the effect of mutations is simply to observe the morphology of mutants at both permissive and restrictive temperatures. As already mentioned, the fact that the 26S proteasome is involved in so many intracellular processes means that pathways involved in the maintenance of cell size and shape might also be affected, resulting in a characteristic morphology.

The cells were grown at both permissive and restrictive temperatures, and the change in morphology was followed over a period of 48hr. The results of this experiment are shown in Figure 6.8.1- 6.8.8. There is a marked difference in the morphology of the *cs* alleles compared to that of the *ts* alleles even at the permissive temperature. Since the *cep2-11* mutation is allelic to *cep2-12*, the *cep-12* mutant was not tested. Since the *cep2* mutants are *cs*, the permissive temperature for these mutants is 30°C, whereas for the *mts2* mutants, it is 25°C.

Wild type *S.pombe* (Figure 6.8.1A and 6.8.5A) are rod-shaped and are approximately 5-10µm in length. By contrast, all of the *mts2* mutants (6.8.2A-6.8.4A) are smaller and more rounded, even at the permissive temperature. This phenotype is similar for all of the *mts* mutants originally isolated. When the *mts2* mutants are transformed with the *mts2*⁺ cDNA, they regain wild-type morphology (data not shown). By comparison, the *cep2* mutants (6.8.6A-6.8.8A) have a more wild type morphology at their permissive temperature.

Incubation of the cells at restrictive temperature (6.8.1B-6.8.8B) produces different morphological changes. All of the *mts2* mutants (6.8.2B-6.8.4B) become elongated, swollen and grossly misshapen. A similar, but less severe phenotype, was observed for *cep2-11* (6.8.7B). This was not the case for *cep2-10* (6.8.6B), and *cep2-13* (6.8.8B), which looked very similar to wild type cells, even after 48 hours of incubation.

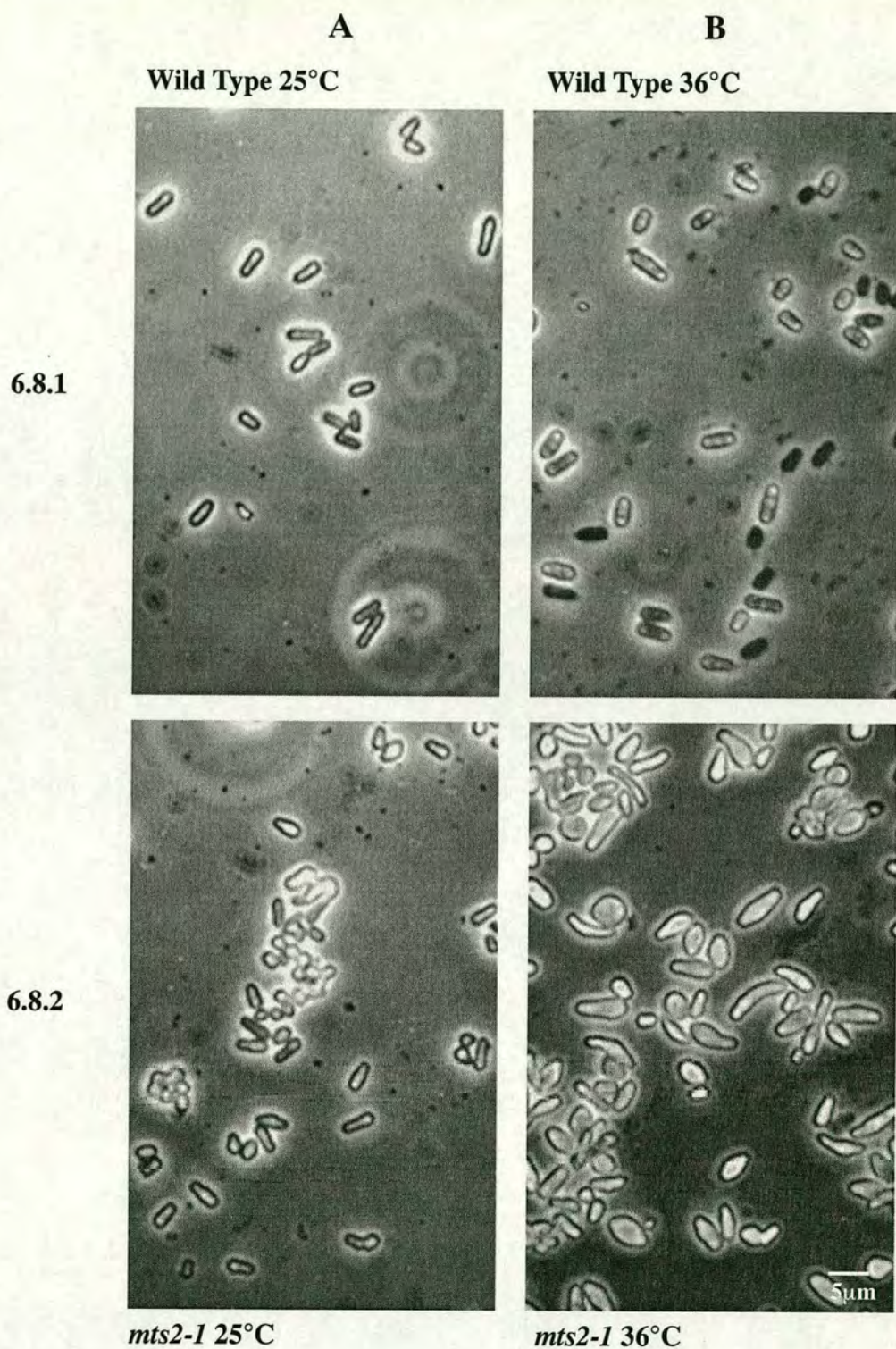
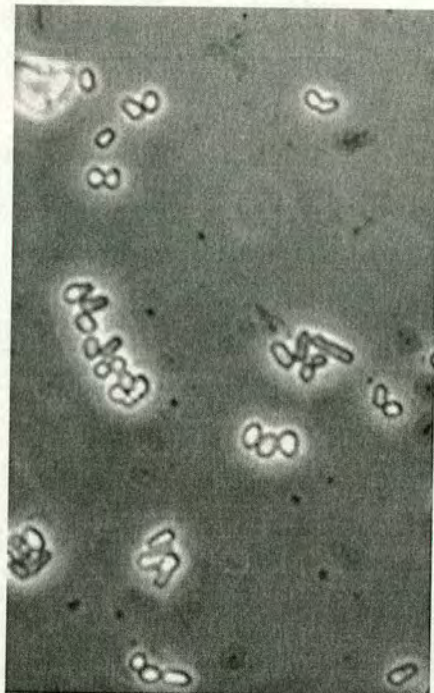


Figure 6.8 Morphology of *mts2* and *cep2* mutants

Asynchronous cultures of all *mts2* and *cep2* strains, incubated at both permissive (left panels) and restrictive (right panels) temperatures, were sampled and examined under light microscopy (x40 magnification). 6.8.1 Wild type *S.pombe*, 6.8.2 *mts2-1*, 6.8.3 *mts2-16*, 6.8.4 *mts2-25* 6.8.5 Wild type *S.pombe*, 6.8.6 *cep2-10*, 6.8.7 *cep2-11*, 6.8.8 *cep2-13*.

A

mts2-16 25°C



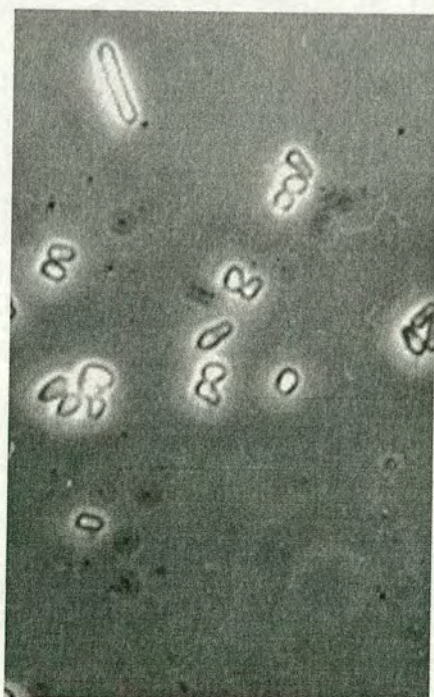
B

mts2-16 36°C



6.8.3

6.8.4

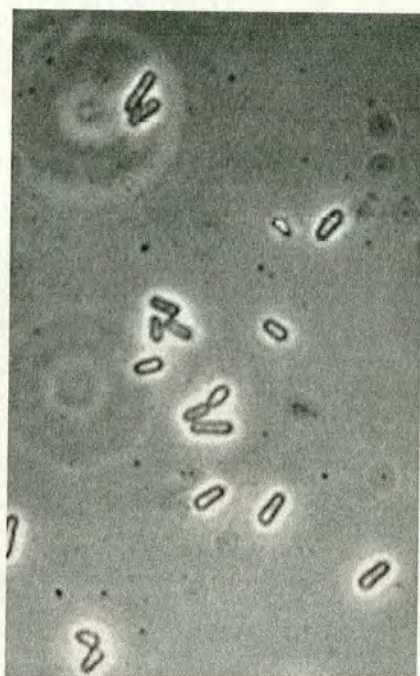


mts2-25 25°C

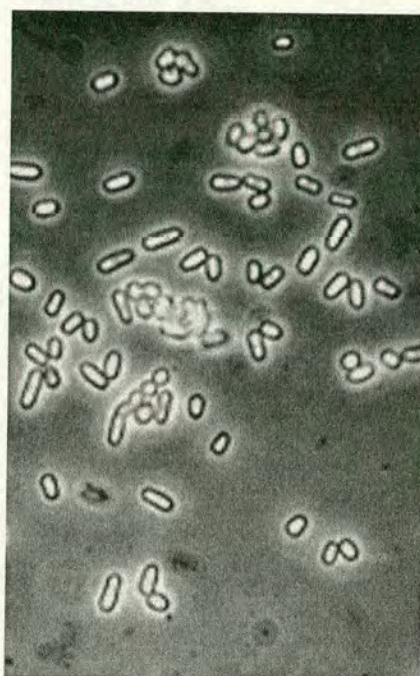


mts2-25 36°C

A
Wild Type 30°C



B
Wild Type 18°C

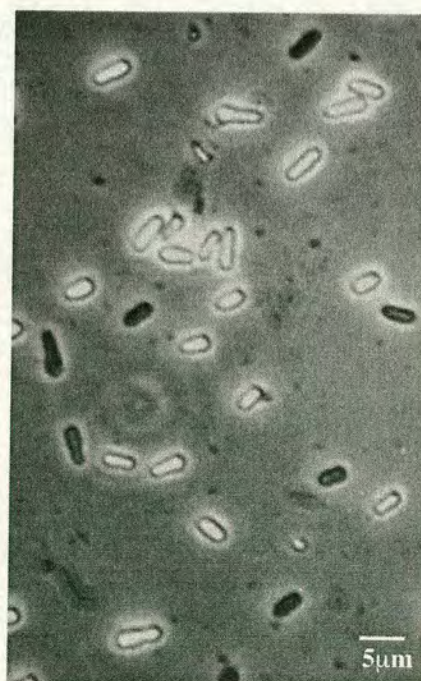


6.8.5

6.8.6



cep2-10 30°C



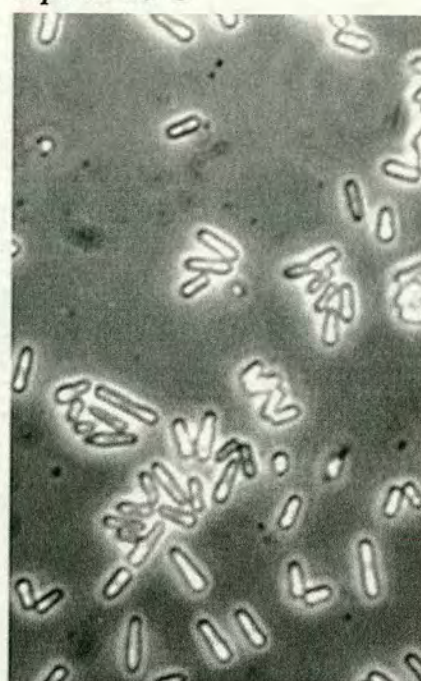
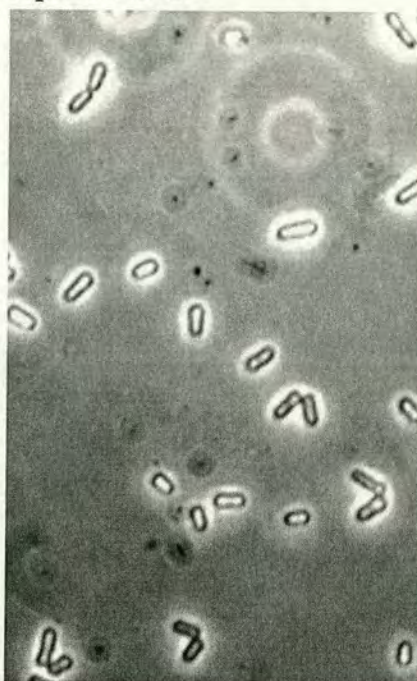
cep2-10 18°C

A

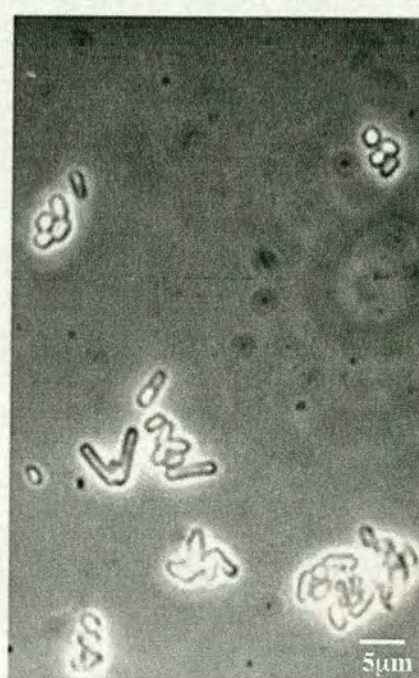
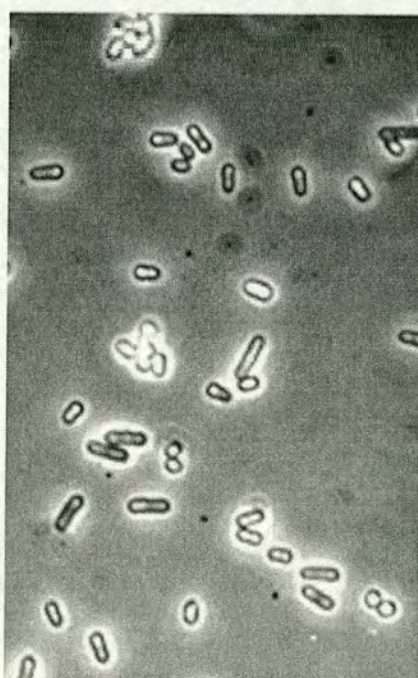
B

cep2-11 30°C

cep2-11 18°C



6.8.7



6.8.8

cep2-13 30°C

cep2-13 18°C

6.5 Temperature sensitivity of *mts2-1*, *mts2-16* and *mts2-25*

As a means of looking at the effect of mutations on the *ts* phenotype of the mutants, the three alleles of *mts2* were streaked onto 5 YE plates and incubated at different temperatures between 25°C and 36°C. The results of this experiment are shown here in Figure 6.9. Most noticeably, *mts2-1* grows at 32°C, but not at 33°C. As discussed in Chapter 3, this precluded the isolation of partial suppressors of *mts2-1*. However, *mts2-25* grows extremely poorly at 32°C, making it more suitable for performing this type of partial suppressor screen. The difference between these two *mts2* alleles, as already described, is the nature of the point mutation. In *mts2-25*, a conserved glycine residue is changed to arginine. This charge difference may destabilise the mutant gene product more than the mutation in *mts2-1*, which is predicted to result in a change from serine to phenylalanine at position 286. Notably, *mts2-16*, which has a change in the codon adjacent to the *mts2-1* mutant, also grows at 32°C.

6.6 Discussion

Mutations in alleles of *mts2* and *cep2* were sequenced and characterised with regard to their effect on their MBC-resistance, morphology and cell-cycle phenotype. The presence of mutations in the *mts2* gene in each case sequenced, in addition to the MBC resistance of all of the alleles, suggests that *cep2*⁺ is the same gene as *mts2*⁺.

The serendipitous manner in which mutations in the *mts2* gene were isolated in two different genetic screens emphasises the ubiquitous function of the 26S proteasome. It is involved not only in the degradation of cell-cycle regulatory proteins, but also in the turnover of proteins which affect centromeric repression, cell morphology and multidrug resistance (MDR).

The mapping of the mutations in both *mts2* and *cep2* alleles highlights the importance of this conserved region of the Mts2 protein. This CAD domain is present in all members of the AAA family of proteins (Confalonieri and Duguet, 1995). Amongst its many predicted functions, this domain, at least in the case of the S4, the human homologue of Mts2p, is known to bind magnesium and ATP (Dubiel *et al.*, 1993). So

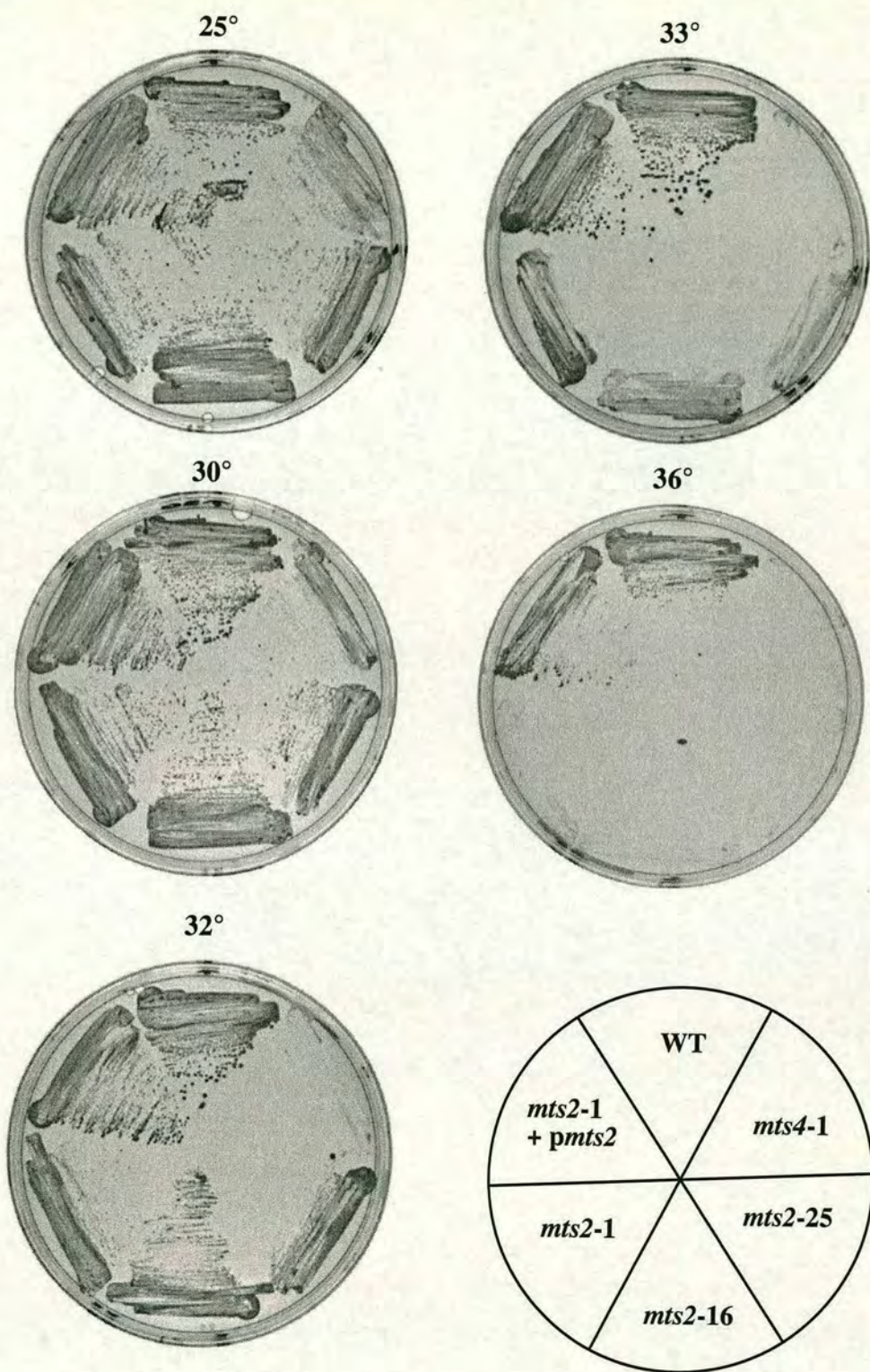


Figure 6.9 Temperature sensitivity of mutant alleles *mts2-1*, *mts2-16* and *mts2-25*

mts2-1, *mts2-16* and *mts2-25* strains were streaked onto complete media and incubated at 25°C, 30°C, 32°C, 33°C and 36°C until colonies had appeared. As controls, *mts2-1* carrying the *mts2*⁺cDNA, and *mts4-1* were used.

far, the S4 subunit is the only proteasomal ATPase that has been directly shown to have ATPase activity (Lucero *et al.*, 1994). Since the peptide sequence of the CAD domain is very similar for all of the proteasomal ATPases, it seems likely that the remaining ATPases also have ATPase activity. However, the 'modulator', a PA700 (19S complex)- dependent activator of the 26S proteasome, which comprises three subunits, of which two are also members of the proteasomal ATPase family, does not have any ATPase activity (Fujiwara *et al.*, 1996).

The complex has specificity for other nucleotides such as GTP and CTP (Hoffman and Rechsteiner, 1996). It is possible that these molecules are bound and hydrolysed by all of the other ATPase subunits, though less efficiently than ATP itself. No mutations have been found in the Walker A motif of the ATPase box (Walker *et al.*, 1982) which bears testament to the essential nature of this motif in Mts2p. However, mutations have been found in this region in the budding yeast homologue of Let1p, another member of the AAA family (Xu *et al.*, 1995).

The mutation in *cep2-10* is consistent with an EMS induced G-A transition at position +871. This results in a change to asparagine from a highly conserved aspartic acid residue at position 291. All other members of the proteasomal AAA family have a aspartic acid residue in this position (see Figure 1.3). It is possible that the cold sensitivity of this mutation occurs as a result of the failure of the *cep2-10* mutant to bind magnesium. An interesting experiment would be to attempt to suppress this mutation by incubating the mutant in or on media which contains elevated levels of magnesium.

The mutation in *mts2-25* is a base transversion from G to C. This is unusual in that it is neither the type of mutation expected following UV nor EMS mutagenesis. Thus it seems that this mutation may have arisen spontaneously. The rate of spontaneous mutation in *S.pombe* is approximately 1×10^{-7} (Moreno and Nurse, 1991). This being so, it is perhaps not surprising that at least one spontaneous mutant has arisen during a screen to look for drug resistant mutants, since drug resistance appears to be mediated by the action of the multi-subunit 26S proteasome complex (M. Penney, pers. comm.).

One interesting result of these experiments is the difference in morphology between these mutants. Although the *cep2* and *mts2* strains are isogenic, there is a morphology difference even under permissive conditions. It might be expected that the genetic background would make a significant difference if mutations affecting cell shape were present. That this is not the case was shown by transformation of *mts2-1* with the *mts2*⁺ cDNA. This completely eradicates the defect in cell morphology of the *mts2-1* mutant indicating that the morphological defect of the *mts2-1* mutant is due solely to a defect in 26S proteasome function even at permissive temperature.

Given that the 26S proteasome is involved in many cellular processes, it is not surprising that the *mts2* mutants have an altered cell shape. However, it might be expected that since the mutations in the *cep2* mutants all map to the *mts2* gene, these mutants would have the same morphology under permissive conditions. At their respective restrictive temperatures, there is an increase in the number of elongated cells which are swollen at one end. This phenotype is seen in all of the proteasomal mutants so far studied and, although the phenotype of the *cep2* mutants is less severe, it is consistent with these mutants carrying a mutation in a gene encoding a subunit of the 26S proteasome. The difference in phenotype also represents a balance between the activity of the proteasome at the permissive and restrictive temperatures. That is that in the *mts2* mutants, proteasome activity, even at the permissive temperature, is lower than that in the *cep2* mutants at their permissive temperature.

The difference in the severity of morphological defects between the *cep2* and the *mts2* mutants is substantiated by their response to different concentrations of MBC. All of the *mts2* mutants grow on MBC concentrations up to 20 µg ml⁻¹ at 25°C, whereas the *cep2* mutants, grown at 30°C because of the *cs* phenotype, are resistant to lower concentrations of MBC. Although it is impossible to compare the two results, due to the differences in temperature at which the experiment was performed, the MBC^R of the *cep2* mutants is nevertheless greater than that of wild type cells. Since resistance to MBC is thought to be mediated through the action of the 26S proteasome (M. Penney, pers. comm.), it follows that the greater the impairment of 26S proteasome function, the more MBC^R will be the mutant. For the *mts2* mutants, the morphology of the cells at the restrictive temperature directly reflects the impair-

ment of 26S proteasome function. This is backed up by staining of the chromosomes and the mitotic spindle.

The lagging chromosome phenotype, seen only in the *cep* mutants, suggests that some cellular function other than progression from metaphase to anaphase may be impaired. As has already been mentioned, the less severe phenotype imposed by the *cep2* mutations may enable completion of the metaphase to anaphase transition, but may prevent the exit from mitosis which requires degradation of the mitotic cyclin Cdc13p. Alternatively, some other function of Mts2p may be impaired. The *cep2-10* and *cep2-13* mutations localise to two regions of the *mts2* gene product which show an extremely high conservation in members of the DEAD-box family of RNA helicases (Gorbalenya *et al.*, 1989). The mutation in *cep2-10* is in the DEID box, which is the equivalent of conserved motif III in RNA helicases (see Figure 7.3), a region involved in RNA unwinding and nucleotide hydrolysis. The mutation in *cep2-13* is in conserved motif IV. If Mts2p does have a role as an RNA helicase, these two mutations may hinder this function. In addition, since the DEID box is known to be involved in chelation of Mg^{2+} ions in ATPases (Walker *et al.*, 1982), a mutation in this region could reduce proteasome function as well as abolishing any potential helicase function. Perhaps it is for this reason that *cep2-10* shows a greater resistance to MBC than either *cep2-13* or *cep2-11/2-12*.

Human Sug1p has been shown to have helicase activity *in vitro* (Fraser *et al.*, 1997). Like Mts2p, Sug1p has been identified as a subunit of the 26S proteasome. Since the *cep* mutants were isolated in a screen to look for enhancers of position effect variegation at the centromere, the potential helicase function of Mts2p may play a role in this phenomenon. If this is so, then this study will have identified at least one other function for some members of the proteasomal ATPase family.

Two other *S.pombe* mutants, *cep1* and *cep3* were isolated in the screen for *cs* mutants. One of these, *cep1*, is an allele of *pad1* (J-P. Javerzat, unpublished result), the gene which encodes a 26S proteasomal subunit and which is involved in the turnover of the Pap1 protein (M.Penney, unpublished result). The identity of *cep3* is as yet unknown but, given that both *cep1*⁺ and *cep2*⁺ encode regulatory subunits of the 26S

proteasome, it is possible that *cep3*⁺ will also turn out to encode a subunit of this complex.

Chapter 7

Chapter 7 General Discussion

7.1 Summary of results from chapters 3-6

- 1) In a yeast 2-hybrid screen, using Mts2p as a bait protein to look for protein-protein interactions, 50 clones were analysed. Of these, 41 carried *let1*⁺, the *S.pombe* homologue of the budding yeast *CIM3/SUG1* gene. From the remaining 12, 6 were found to encode *aps1*⁺, the *S.pombe* homologue of the budding yeast *CIM5* and the mouse MSS1 gene. Like *mts2*⁺, these genes encode members of the AAA family of ATPases which are also components of the 26S proteasome (Confalonieri and Duguet, 1995).
- 2) A 2-hybrid assay for interactions between Mts2p, Let1p and MSS1p demonstrated that whereas Mts2p interacted with both Mss1p, Let1p and itself, neither Mss1p nor Let1p interacted with themselves or with each other.
- 3) The isolation of a truncated *let1* gene during the 2-hybrid screen suggested that the N-terminal 33 amino acid residues of Let1p were dispensable for the interaction between Mts2p and Let1p. This was confirmed by deletion analysis of both proteins. Furthermore, it was shown that the essential domain for this interaction was not contained within the N-terminal 72 amino acid residues of Let1p. The N-terminal 106 amino acid residues of Mts2p were sufficient to bind to Let1p in this system.
- 4) A homotypic interaction between Mts2p and itself required sequences within the conserved ATPase domain of the protein.
- 5) The over-expression of C-terminally truncated versions of Let1p had a dominant negative effect..

6) The *let1*⁺ gene was found to be essential for growth, since a haploid strain containing a disrupted copy of the *let1* gene was non-viable and resulted in an increase in the number of cells containing condensed chromosomes and a short mitotic spindle. This transient phenotype gave rise to a large number of cells which were septated but in which nuclear division had not taken place. FACS analysis indicated that prior to arrest of the cell cycle with a short mitotic spindle, these cells had undergone DNA replication. This phenotype is very similar to that observed for disruptions in genes encoding other regulatory subunits of the 26S proteasome.

7) Mutation analysis of 7 alleles of the *mts2* mutant, isolated by 2 different screens, revealed a clustering of mutations within the CAD region of Mts2p. Although none of these mutations were found in the ATP-binding domain of the protein, one lay in the magnesium binding domain (*cep2-10*) and the remainder were found in conserved residues within the CAD region. Two of the mutations, *cep2-10* and *cep2-13*, lie in motifs conserved in a family of RNA/ DNA helicases.

8) A comparison of MBC resistance and morphology at restrictive temperatures showed that the *ts* mutants (*mts2-1*, *2-16* and *2-25*) all had a more severe phenotype at their restrictive temperature as compared to the *cs* mutants (*cep2-10*, *2-11*, *2-12* and *2-13*).

7.2 Let1p: 26S proteasomal subunit, transcriptional regulator or both?

Prior to the commencement of this work Sug1p, the budding yeast homologue of Let1p, was thought to be solely a modulator of transcription. This was based on its isolation as an extragenic suppressor of a C-terminal deletion of the Gal4 transcription factor in budding yeast (Swaffield *et al.*, 1992). This theory was strengthened by the discovery of Sug1p in an RNA Polymerase II holo-enzyme complex (Kim *et al.*, 1994). Other work has implicated *SUG1* variously as a modulator of the transcription factor encoded by the *CDC68* gene in budding yeast (Xu *et al.*, 1995) as well as a mediator of the ligand-dependent transcriptional activity of nuclear receptors (vom

Baur *et al.*, 1996). By contrast, the results presented in chapters 3-5, as well as by other groups (Rubin *et al.*, 1995; Sun *et al.*, 1996; Wang *et al.*, 1996), suggest that Let1p, and all of its orthologues, function primarily as subunits of the 26S proteasome.

Sug1p was found to be associated with the RNA polymerase II holo-enzyme complex, a transcriptional mediator complex which activates both basal and activated transcription *in vitro* (Kim *et al.*, 1994). This discovery suggested that either Sug1p was a multi-functional protein, which would support the theory that it was involved in transcription, or that this occurrence was due to contamination of the holo-enzyme preparation with the Sug1 protein. In the 26S proteasome, Sug1p has been shown to be present at a ratio of 1:1 Sug1p:26S proteasome (Rubin *et al.*, 1996). When the amount of Sug1p in the holo-enzyme complex was measured, relative to the other components, it was found to be present in a ratio of 0.05:1. In addition, Sug1p has not been found in preparations of the holo-enzyme complex from other laboratories, suggesting that contamination of the extracts with Sug1p had occurred (Rubin *et al.*, 1996)

Reports detailing a number of 2-hybrid interactions between homologues of Let1p and other nuclear receptors and transcription factors such as the thyroid hormone receptor (Lee *et al.*, 1995), retinoic acid receptor α (RAR α) (vom Baur *et al.*, 1996), GAL4 and TATA-binding protein (Swaffield *et al.*, 1995; Melcher and Johnston, 1995) suggest an association between Let1p homologues and many different types of protein molecule at least in the 2-hybrid assay system. Although many of the above molecules have a common function, it is worth noting that subunits of the 26S proteasome are among the most commonly isolated 'false positives' during 2-hybrid library screening (Hengen, 1997), suggesting that some of the interactions described above may be non-specific.

The *sug1-20 / 1-26* mutation was found to suppress the *ts* phenotype of the *cdc68-1* mutant of *S.cerevisiae* in an allele-specific manner (Xu *et al.*, 1996). The mutation lies in the Walker-A motif of the ATP-binding site in the *SUG1* gene product (Walker *et al.*, 1982). This is a different mutation from that of *sug1-1* which restores

transactivation function to the mutant Gal4 protein encoded by the *gal4D* allele (Swaffield *et al.*, 1992). Both of these mutations, in addition to *sug1-3*, which also lies in the conserved ATPase domain, contribute to the stability of the mutant Cdc68 protein. The nature of the suppression suggests an interaction between the ATPase domain of the Sug1 protein and the Cdc68 protein. The increased stability of the mutant Cdc68 protein in a *sug1* background, and thereby increased transcription of Cdc68p target genes, can be explained by the reduced ability of the 26S proteasome to degrade Cdc68p.

The isolation of the gene encoding Let1p in the yeast 2-hybrid screen, using Mts2p as a bait protein, was unexpected. However, the homology of the peptide sequence to that of other ATPase subunits of the 26S proteasome, suggested that there may be an *in vivo* interaction between these 2 subunits. Significantly, a disruption of the *let1*⁺ ORF by insertion of the *ura4*⁺ gene confirmed that like all but 1 of the genes encoding regulatory subunits of the 26S proteasome, it was essential for cell viability. Also, the cytological phenotype of this disruption was very similar to that observed for a disruption of *mts2*⁺ (C.Gordon, unpublished results) which encodes subunit 4 of the 26S proteasome, *mts3*⁺, which encodes subunit 14 (Gordon *et al.*, 1996) and for a disruption of *mts4*⁺ which encodes subunit 2 (Wilkinson *et al.*, 1997).

The presence of Let1p in the *S.pombe* 26S proteasome was confirmed by western blotting of purified preparations of the 26S complex with an antibody raised against Cim3p/Sug1p (Wolfgang Dubiel, unpublished result, and see Figure 5.11). A similar result was shown in *S.cerevisiae* (Rubin *et al.*, 1996). These results support those in Chapter 3 which demonstrate a possible interaction between Let1p, Mts2p and MSS1p (Figure 3.12). Furthermore, the suppression of a mutation in an α -type subunit of the 20S proteasome by a mutation in Sug1p (U-M. Gerlinger, unpublished result), provides further evidence that Sug1p/Let1p is a component of the 26S proteasome.

Despite all the evidence confirming the presence of Let1p/Sug1p in the 26S proteasome, there is as yet no direct evidence to suggest a specific *in vivo* interaction between Let1p and the product of the *mts2*⁺ gene. Unlike *aps1*⁺, the murine homologue

of which, MSS1, was shown to rescue the *ts* defect of the *mts2-1* mutant when over-expressed (Gordon *et al.*, 1993), over-expression of *let1*⁺ failed to rescue *mts2-1*. Experiments to demonstrate *in vitro* interactions by binding assays using epitope-tagged and bacterially expressed Mts2p and Let1p, and immunoprecipitation, have so far been unsuccessful.

The proteasomal subunit / transcriptional modulator paradox can be addressed by proposing that Let1p and its orthologues, as well as other 26S proteasomal subunits, possess the ability to recruit molecules destined for proteolytic degradation. This would account for the observed *in vitro* associations between Sug1p and transcriptionally associated molecules such as the human thyroid hormone receptor (Lee *et al.*, 1995) c-Fos (Wang *et al.*, 1996), RXR (vom Baur *et al.*, 1996) and RAR α . Like eukaryotic proteins such as p53, c-Myc etc., transcriptional regulators are themselves presumably short-lived *in vivo*. The recruitment of these proteins directly by independent subunits of the 26S proteasome, followed by their subsequent degradation, would prevent an intracellular accumulation of these molecules.

Phenotypic differences among mutations in genes encoding proteasomal subunits, and more particularly ATPases, might reflect the substrate specificity of these proteasomal subunits. Disruptions of the genes encoding Mts2p (C. Gordon, unpublished results) and Let1p (this work), in fission yeast, and Cim5p/MSS1p (Ghislain *et al.*, 1993) in budding yeast, result in a phenotype characterised by a defect at the metaphase to anaphase transition. This result is thought to reflect the requirement for the proteasome to degrade some protein(s) which holds sister-chromatids together (Holloway *et al.*, 1993). A recent result showed that Sug2p, the sixth proteasomal ATPase subunit (de Martino *et al.*, 1996; Russell *et al.*, 1996), is required to mediate spindle pole body segregation in budding yeast, a stage earlier than that associated with the action of Mts2p and Let1p (McDonald and Byers, 1997). Since this is a point mutation, it may therefore not reflect the phenotype of a disruption of the *SUG2* gene.

Whereas the above-mentioned ATPases seem to be primarily proteasomal in function, a number of results suggest that TBP-1, (S6') of the proteasome (Richmond *et*

al., 1997), may have more than just a proteasomal function. Firstly, this subunit was isolated as binding to the human HIV transactivator Tat (Nelbock *et al.*, 1990). Secondly, an over-expression of this subunit in *S.cerevisiae* is lethal (Schnall *et al.*, 1994) whereas over-expression of other ATPases such as Let1p in fission yeast is not lethal. Thirdly, a disruption of the homologue of TBP-1 from *Dictyostelium discoideum* did not result in the transient metaphase arrest that has been associated with disruptions in other proteasomal ATPases. Instead of this, large multinucleated cells were formed (Cao and Firtel, 1995). Finally, whereas other proteasomal ATPases such as S4 (Mts2p), MS73 (TBP-7) and MSS1 were co-ordinately up-regulated during degradation of the inter-segmental muscles of the tobacco hawkmoth *Manduca sexta*, the level of TBP-1 was at a constitutively high level (Dawson *et al.*, 1994; Jones *et al.*, 1995). These results suggest that TBP-1 and its homologues may be regulated in a manner independent of other proteasomal subunits, and that the constitutively high level of this protein could reflect its involvement in a variety of cellular processes such as transcription.

All of the evidence presented above points to Let1p/Sug1p as being primarily a subunit of the 26S proteasome. The requirement for cells to degrade short-lived proteins such as transcription factors and transcriptional regulators, can be used to explain the interaction of Sug1p/Let1p with these types of molecules. The presence of Sug1p in proteasomes from mammalian red blood cells, which are anucleate and which therefore don't undergo any gene expression, suggest that this is the main function of Sug1p (Dubiel *et al.*, 1994). This notwithstanding, it is still possible that a molecule such as TBP-1 (S6') may be involved in regulating more than just proteolysis.

7.3 Structure, assembly and interactions between subunits of the ATPase ring of the 26S proteasome

EM pictures and electron density maps of the 20S proteasome have shown that this structure is formed by 4 heptameric rings, of which the 2 centre rings comprise β subunits and the 2 outer rings comprise α subunits (Peters *et al.*, 1994, and see Figure

1.2). In the centre of these 4 sets of rings is a narrow channel which is thought to enable the passage of substrate proteins into the catalytic chamber formed by the β subunits, where they are subsequently degraded. In keeping with this structure, and by analogy to the ClpP/ClpX protease of *E.coli* (see Chapter 1), it was postulated that the ATPases of the 19S regulatory complex might also form a ring to enable channelling of an unfolded substrate through the 26S complex. The remainder of the 0. regulatory subunits then bound 'on top of' the ATPase ring to give the 26S proteasome its characteristic 'caterpillar' shape (Yoshimura *et al.*, 1993). Although some of the interactions between these ATPase subunits has been described (Chapters 3-5), the remainder of known interactions are described below. Despite knowledge of these interactions, however, neither the localisation or characterisation of all of the subunits of the 19S complex is yet complete.

7.3.1 Inter-subunit interactions

7.3.1.1 Interactions within the 26S proteasome

It was originally proposed that there were only 4 proteasomal ATPases which formed a ring on top of the 20S complex (Dubiel *et al.*, 1993). These subunits, based on the work presented in Chapter 3, were thought to be Mts2p (S4), MSS1p/Cim5p/Aps1p (S7), Sug1p/Let1p (S8), and a homologue of a further ATPase subunit, S6 (TBP-7) (Dubiel *et al.*, 1994). More recent work has confirmed the presence of 2 more ATPases, TBP-1 (S6') and Sug2p (S10b) in the 26S proteasome (De Martino *et al.*, 1996). Thus, in total, there are 6 ATPases within the 19S regulatory complex of the 26S proteasome which are believed to be arranged in a ring conformation (Hershko and Ciechanover, 1992; Dubiel *et al.*, 1995).

TBP-1 was isolated from a human cDNA expression library using biotinylated Tat as a probe. TBP-7 was isolated on the basis of its homology to TBP-1 (Ohana *et al.*, 1993). The proteins encoded by these genes were thought to be transcriptional activators because of their binding to Tat, a transactivator of the human HIV gene expression. The presence of both of these proteins in the 26S complex has now been

confirmed (Dubiel *et al.*, 1994; Richmond *et al.*, 1997). The interaction between these two proteins appeared to be mediated by the N-terminus such that the N-terminal 100 amino acid residues were necessary and sufficient for this interaction (Ohana *et al.*, 1993). Using a 2-hybrid screen, TBP-7 was found to bind to a nuclear hormone receptor and to the human homologue of MSS1p (Choi *et al.*, 1996).

SUG2 has been isolated from erythrocytes, yeast cells, ground squirrel and from the tobacco hawkmoth *Manduca sexta* (Bauer *et al.*, 1996; Fujiwara *et al.*, 1996; Russell *et al.*, 1996; Dawson *et al.*, 1997). In mammalian cells, Sug2p was also found to be present in a secondary complex, termed the 'modulator', along with TBP-1 (de Martino *et al.*, 1996; Fujiwara *et al.*, 1996 and see section 7.3.2). An association between Sug2p and Sug1p has been demonstrated by a two hybrid approach and genetically by a demonstration of synthetic lethality between the *sug1-1* and *sug2-1* mutants (Russell *et al.*, 1996).

From the work presented earlier, we believe that the subunit encoded by *mts2*⁺ lies between those encoded by *aps1*⁺ and *let1*⁺. MSS1p, a mammalian homologue of Aps1p has been shown to interact with TBP-7 using a 2-hybrid approach (Choi *et al.*, 1996). The interaction between TBP-1 and TBP-7 was also demonstrated by a 2-hybrid approach (Ohana *et al.*, 1993). Sug1p has been shown to interact with Sug2p both by 2-hybrid analysis and synthetic lethality studies (Russell *et al.*, 1996). As previously mentioned TBP-1 is present in the 'modulator' with Sug2p, indicating that this interaction occurs *in vivo*, and presumably in the 26S proteasome.

Given the interactions described above, it is possible that the ATPase ring is formed in the manner shown in Figure 7.1. It has not yet been determined whether different ATPases are present in different subsets of proteasomes, but given the stoichiometry of the subunits isolated from human proteasomes, this seems unlikely (Dubiel *et al.*, 1993).

An alternative arrangement of ATPases has been proposed (Richmond *et al.*, 1997). This model proposes that the ATPases are arranged in a 4-membered ring with the

PROPOSED ARRANGEMENTS OF ATPases IN 26S PROTEASOME

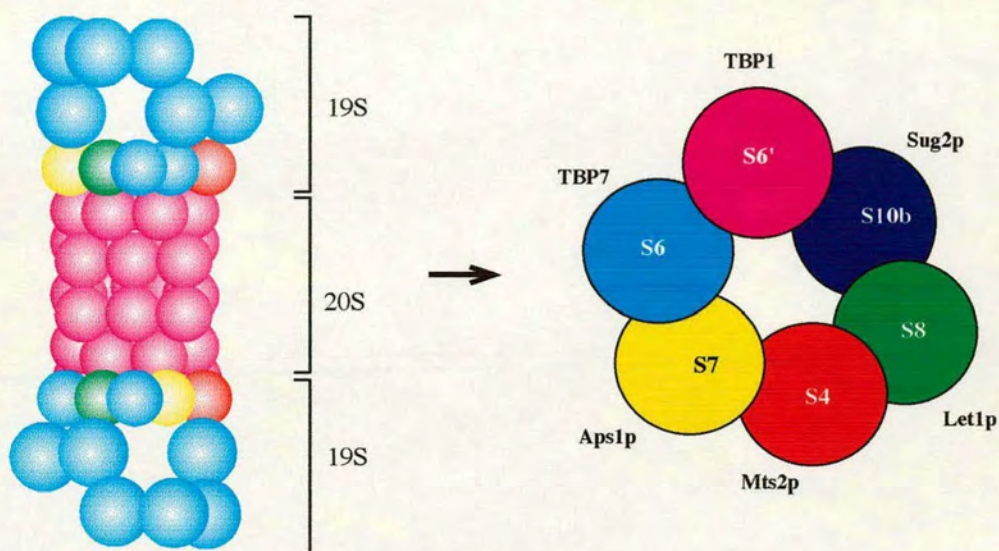


Figure 7.1 Proposed arrangement of ATPases in the 26S proteasome

The six ATPase subunits of the 26S proteasome are annotated according to Richmond *et al.*, 1997

remaining two subunits, Sug2p(S10b) and TBP-1 (S6'), being loosely associated on the periphery of this ring. Using an *in vitro* binding assay an overlay assay, no interaction was detected between S10b and S6' and any other ATPases. By contrast, S4 formed a quaternary complex *in vitro* with S6, S7 and S8.

7.3.1.2 Assembly of the ATPase ring

The results from Chapter 4 and from work on other ATPase subunits (Ohana *et al.*, 1993; Richmond *et al.*, 1997), suggest that the N-terminus of each subunit mediates an inter-subunit interaction, whereas the C-terminus is dispensable for this function. It is therefore possible that all of these subunits assemble in the same way by an association of N-termini. This would leave the conserved C-terminal domain free to either bind proteolytic substrates, assist in potential unfolding of these substrates and formation of the 26S complex, through ATP hydrolysis in the case of Mts2p, or to fulfil some other as yet unknown role associated with the proteasome.

A model for the assembly of the ATPase ring is shown in Figure 7.2. This model proposes firstly that the N-termini of subunits interact to form dimers or trimers and that the pairing of two trimers or three dimers in the correct configuration, results in formation of the ATPase ring. Furthermore, given the interaction of Sug1p with c-Fos and other nuclear receptors *via* the N-terminus, which contains the coiled-coil motif, or ATPase domain, this model assumes that this may be a method to recruit substrates for degradation. Interaction and localisation of N-termini could be directly tested either by epitope-tagging, or by raising antibodies against these domains and observing the staining which appeared. If no staining was visible, it would suggest that the N-terminus of each subunit was present on the inside of the ATPase ring, whereas staining on the outside of the particle would suggest that the N-termini faced the cytoplasm, which may help to explain the association of ATPases with substrate molecules (Wang *et al.*, 1996)

7.3.1.3 Assembly of the 26S complex

In addition to its interactions with other ATPase subunits, Mts2p has been found to interact with Mts4p, a non-ATPase subunit (Wilkinson *et al.*, 1997). Like Let1p, this interaction may be mediated by sequences within the N-terminus, since deletion of the C-terminus of Mts2p, up to and including the ATPase domain, has no effect on binding of Mts4p (C. Wilkinson, unpublished results). This is the first demonstration that the ATPase 'ring' of the 26S proteasome interacts with non-ATPase subunits of the 19S regulatory complex.

What of the interaction between the 19S and the 20S complexes? As mentioned, there has been a report of suppression of the cycloheximide resistant *crl3-2* mutant of *S.cerevisiae* (McCusker and Haber, 1988) by a mutation in the gene *SCL1* (suppressor of *crl3-2* lethality) (Balzi *et al.*, 1989). *SCL1* encodes an α -type subunit of the 20S proteasome, and *crl3-2* is a mutation in the gene encoding the budding yeast homologue of Let1p (U-M. Gerlinger, unpublished result). This is the first demonstration of a genetic interaction between these two complexes. From the original MBC^R screen (Gordon *et al.*, 1993), *ts* mutations in *S.pombe* genes encoding 2 of the 7 α -type subunits of the 20S proteasome have been isolated (O. Rooyackers, M. Wallace, C. Wilkinson and C. Gordon, unpublished results). Reversion analysis of these mutants may reveal as yet unidentified interactions between the 19S and 20S complexes.

The results of the 2-hybrid screen, described in Chapter 3, showed that no 20S subunits had been isolated. One explanation for this is that assembly of the two complexes is only possible when both have assembled independently. It is known that the 20S complex associates from two identical halves (Chen and Hochstrasser, 1995, 1996). It may be that the conformation of individual 19S subunits is altered by their interaction, thereby facilitating their binding to the α subunits of the 20S complex. A knowledge of the crystal structure of the ATPase subunits would enable the introduction of site-specific mutations in potential 19S/20S contact points, which may suppress the conditional lethality of those mutations already isolated in the 20S

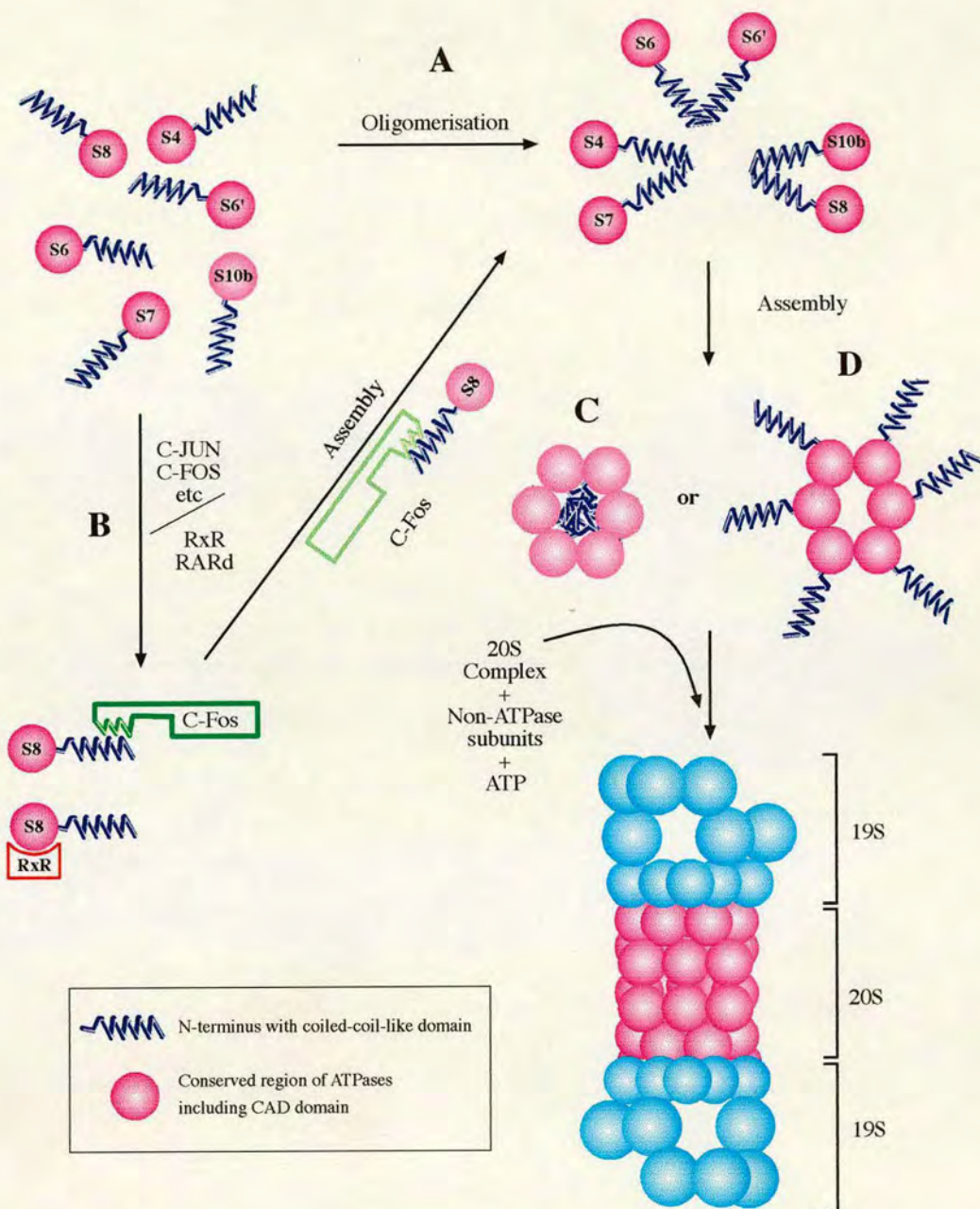


Figure 7.2 Schematic model for the recruitment of substrates of the 26S proteasome by independent subunits and biogenesis of the ATPase ring

Individual subunits could either oligomerise without recruitment of (A) or following recruitment of (B) substrates destined for degradation by the 26S proteasome. Interaction between subunits, and between subunits and targets may be facilitated by the N-terminal region of the molecules. This model makes no prediction as to the state of ubiquitination or phosphorylation of target molecules. Following oligomerisation, an ATPase ring could assemble with N-termini internal (C) or external (D) to the ATPase ring. Once assembly has taken place, other subunits may be added to form the 19S regulatory complex.

subunits. An alternative explanation may be that the use of ATPases fused to the *GAL4* DNA-binding domain in the vector pAS2 (see Materials and Methods) prevents an interaction between ATPases and α subunits either by changing the conformation of the ATPase or by steric hindrance of the interaction by the Gal4 domain.

7.3.2 Other interactions between proteasomal ATPases

In addition to being found in the 26S proteasome, two ATPases, TBP-1 (S6') and Sug2p (S10b) have been found, along with a third protein, in a complex termed the 'modulator' (De Martino *et al.*, 1996; Fujiwara *et al.*, 1996). This complex was isolated by its ability to stimulate the peptidase activity of the 26S proteasome 8-fold. Despite possessing two ATPases, the complex has no endogenous ATPase activity *in vitro*. It is possible that this complex acts to recruit a 19S complex to both ends of the 20S proteasome thereby forming a ternary complex. If this was the case, it might be expected that this complex would also be found in other organisms.

7.4 Possible roles of the proteasomal ATPases

7.4.1 ATPases as chaperones / anti-chaperones. Comparison with ClpAP/ClpXP/hslUV and Lon proteases of *E.coli*

Molecular chaperones catalyse the refolding of denatured proteins which occurs after stresses such as high temperature. When proteins misfold, chaperones are thought to assist in the removal of proteins by proteolytic degradation. This is done by preventing the formation of aggregates and by targeting the proteins to the intracellular proteolytic complexes (Hayes and Dice, 1996). In *E.coli*, a major group of chaperones are induced following heat-shock. Another group of up-regulated proteins are proteases which catalyse the selective degradation of the misfolded proteins. These include the ATP-dependent proteases Lon, ClpP and HslVU (Rohrwild *et al.*, 1996). The association of a multimeric ATPase complex with ClpP leads to the formation of the ClpAP and ClpXP ATP-dependent proteases.

The proposal for the anti-chaperone action of the hexameric ATPase ring of the 26S proteasome owes much to its similarity to the structure of the ClpAP/ ClpXP/ HslVU/ Lon proteases of *E.coli*. Firstly, the symmetry of these prokaryotic complexes mirrors that of the 20S complex in that ClpP, the equivalent of the 20S proteasome has 7-fold symmetry, whereas ClpA and ClpX which form a ring of ATPases, have 6-fold symmetry (Schirmer *et al.*, 1996). The addition of these two separate ATPase complexes to the catalytic core of ClpP, confers different substrate specificity on the respective complexes (Levchenko *et al.*, 1995). The symmetry of the HslVU protease is slightly different in that HslV, which has homology to the β subunits of the 20S proteasome (Rohrwild *et al.*, 1996) has 6-fold symmetry (Missiakos *et al.*, 1996).

X-Ray crystal structure data obtained for the budding yeast 20S proteasome suggests that the complex has no pore at either end (Groll *et al.*, 1997). Passage of unfolded proteins into this complex is thought to be facilitated by the 'capping' of the 20S complex by the 19S or 'modulator' complex which mediates opening of a central channel. This being so, one requirement prior to proteolysis is the complete unfolding of substrate polypeptides. One way in which this could be achieved would be by binding of ATPases to substrates, which, if denatured, would prevent their aggregation or, if folded, might result in the partial or full unfolding of the substrate. This ability to unfold substrates mirrors the action of the Clp A/X proteins, which are members of the HSP100 family of chaperones (Hayes and Dice, 1996; Schirmer *et al.*, 1996). The unfolding of substrates may require energy in the form of ATP hydrolysis.

In the bacterial ClpP protease, proteolysis of small substrates requires neither ATP-binding nor ATP hydrolysis (Rohrwild *et al.*, 1996). By contrast, the proteolytic activity of the HslVU protease is stimulated by the addition of ATP (Yoo *et al.*, 1997). The ATPase component of this proteolytic complex, HslU, can form ring-shaped particles in the presence of ATP, ADP and AMP-PNP, indicating that nucleotide binding rather than ATP hydrolysis is required for oligomerisation of HslU (Rohrwild *et al.*, 1997). However, ATP hydrolysis by HslU is coupled to proteolysis

by HslV, since non-hydrolysable analogues of ATP inhibit proteolysis (Yoo *et al.*, 1996). ATP consumption may be linked to formation of the active enzyme, or to peptide hydrolysis, as is the case for the 26S proteasome (Rohrwild *et al.*, 1996).

The ATPase ring, and more specifically the S4 subunit, has been shown to have ATPase activity (Lucero *et al.*, 1994). In addition, the 26S proteasome demonstrates a broad nucleotide specificity in that CTP, GTP and UTP can also be hydrolysed, indicating that some members of the proteasomal S4 family may provide energy by the hydrolysis of nucleotides other than ATP (Hoffman and Rechsteiner, 1996).

Members of the AAA family also function as chaperones and proteolytic machines. The YTA10-YTA12 ATPases of budding yeast, located in the mitochondrial inner-membrane, are homologous to the prokaryotic FtsH protein of *E.coli* and form a proteolytic complex (Arlt *et al.*, 1996; Leonhard *et al.*, 1996). The chaperone/ anti-chaperone activity of these molecules is independent of the proteolytic activity. It is thought that ATP is required for formation of the YTA10-YTA12 complex in addition to the unfolding of potential proteolytic substrates. This situation is similar to that envisaged for the ATPases of the 26S proteasome.

7.4.2 ATPases as RNA/DNA helicases

In addition to possessing the two motifs required for ATP binding and hydrolysis, all of the proteasomal ATPases possess other motifs which are common to a family of RNA/ DNA helicases known as the DEAD-box family (Gorbalenya *et al.*, 1989). These motifs, shown in Figure 7.3, are essential for RNA unwinding (Schmid and Linder, 1992). A comparison of the proteasomal ATPases shows that all of them possess a similar spacing between the motifs thought to be involved in this cellular function (Makino *et al.*, 1996).

Sug1p was recently shown to exhibit 3'-5' DNA helicase activity which was abolished by a mutation in the ATP-binding domain (Fraser *et al.*, 1997). This finding implicates Sug1p and perhaps the remaining ATPases with a function in RNA/DNA

metabolism. Sug1p shares homology with the RuvB protein from *E.coli*, which is a Holliday junction helicase involved in bacterial recombination (Fraser *et al.*, 1996). Taking into account that a number of these ATPases :- TBP-1, TBP-7, MSS1 and Sug1p were thought to be involved in transcription (Nelbock *et al.*, 1990; Shibuya *et al.*, 1992; Swaffield *et al.*, 1992; Ohana *et al.*, 1993), and that they all possess the motifs common to DEAD-box helicases, it is possible that they function as helicases in the mediation of transcription. Alternatively, it has been suggested that they may help to remove ubiquitinated proteins, such as histones, from DNA, thus facilitating chromosome condensation during mitosis (Fraser *et al.*, 1997).

7.4.3 ATPases in the recruitment of proteasomal substrates

The possible recruitment of proteolytic substrates by Sug1p has already been discussed (see 7.3.1 and Figure 7.2). This function may be mediated by the presence on at least 4 of the 6 ATPases of a leucine-zipper motif, known to be involved in protein-protein interactions (Johnson and McKnight, 1992). Although missing the basic DNA-binding domain common to members of the bHLH/Zip family such as c-Jun and c-Fos, the leucine-zipper motif has been shown firstly to mediate inter-subunit interactions (see Chapter 5) and to mediate the interaction between Sug1p and c-Fos (Wang *et al.*, 1996).

If the individual subunits are involved in the recruitment of proteolytic substrates, there must be some mechanism for 'pushing' the bound substrates into the catalytic core of the 20S proteasome following assembly of the 26S complex. Since even partially folded substrates are too large to pass through the pore at either end of the catalytic core, the unfolding of the substrate may be the result of a co-operative interaction between the other ATPase subunits of the proteasome. Initial binding of a substrate, such as c-Fos, by Sug1p may be the initial step in a conformation change which is required for attachment of polyubiquitin chains prior to degradation. The binding of polyubiquitin by S5a (Deveraux *et al.*, 1994) may serve as an anchor point for the substrate which is then effectively unfolded by binding to other neighbouring proteasomal ATPases. Processive unfolding by these ATPase 'anti-chaperones' could

be linked to entry of the substrate into the proteolytic core. This 'ratchet' model for the action of the proteasomal ATPases is analogous to the situation observed in the F_1 -ATPases complex of the F_0 - F_1 ATP synthase (Abrahams *et al.*, 1994). In this ATPase complex which comprises 3 α and 3 β subunits, the 3 β ATPases possess catalytic sites which are in different states of the catalytic cycle. The inter-conversion of these states is thought to be achieved by the rotation of the 6 ATPases relative to an α -helical domain of a γ -subunit. Thus, in the same way, binding of the polyubiquitin moiety of a proteolytic substrate by either S5a or another proposed ubiquitin-binding subunit (van Nocker *et al.*, 1996), followed by rotation of the ATPases, could facilitate unwinding of the proteolytic substrate.

7.5 The *mts2* mutants: Genotype versus Phenotype

Chapter 6 describes the mutation analysis and phenotype of 6 independent mutations in the *mts2* gene. All of these mutations occur in the conserved region of the gene, giving rise to altered amino acids in the mutant protein which in each case result in a conditional phenotype. Figure 6.4 shows that most of these mutations do not lie in any recognisable motif, and are thus likely to have an effect solely on the structure of the mutant subunit. However, the mutations in the *cep2-10* and *cep2-13* alleles lie in two recognisable motifs. *Cep2-10* lies in the DEID box motif, a region thought to be involved in anion-dependent nucleotide hydrolysis. As mentioned in section 7.3.3, this motif is also thought to be essential for the RNA / DNA unwinding capabilities of members of the DEAD-box helicase family (Gorbalenya *et al.*, 1989). The mutation in *cep2-13* lies in conserved motif IV, also present in this family of helicases. The presence of these mutations raises the possibility that Mts2p, as well as being involved in the function of the proteasome, also possesses helicase activity like Sug1p (Fraser *et al.*, 1997).

The contribution of all of the mutations in the *mts2* gene to MBC resistance, mediated by the 26S proteasome (M. Penney, unpublished results), was measured. In each case, the mutants isolated in the original MBC screen (Gordon *et al.*, 1993) turned out to be more resistant to MBC than the *cep2* alleles. This was a test of proteasome

MOTIF	I	II	III	IV
Mts2	GAPGTGK	DEID	MAT	LRPGRIDR
Mss1	GPPGTGK	DEID	FAT	MRPGRIDR
Tbp1	GPPGTGK	DELD	AAT	LRSGRLDR
Tbp7	GPPGcGK	DEID	MAT	LRPGRLDR
Sug1	GPPGTGK	DEID	MAT	LRPGRIDR
Sug2	GPPGTGK	DEVD	MAT	LRPGRLDR
CONSENSUS	GxPGxGK	DExD	xAT	xRxxRxxR

Motif I	Nucleotide binding
Motif II	Interaction with Mg^{2+} , coupling of ATP hydrolysis to RNA unwinding (Mian <i>et al.</i> , 1993)
Motif III	RNA unwinding (Pause and Sonnenberg, 1992)
Motif IV	RNA unwinding

Figure 7.3 Comparison of RNA / DNA helicase motifs in proteasomal ATPases

function, and suggested that in the *mts2* mutants, the level of proteasome activity, compared to that in the *cep2* mutants, was reduced.

The phenotype and morphology of the *cep2* alleles was distinct from that of the *mts2* alleles in that for the *cep2* alleles, lagging chromosomes along the anaphase spindle were visible (J-P. Javerzat *et al*, manuscript in preparation) and cell morphology appeared normal. All of the *mts2* mutants displayed the metaphase arrest phenotype characteristic of mutations in regulatory subunits of the 26S proteasome of fission yeast (Gordon *et al.*, 1993, 1996; C. Wilkinson, unpublished results; this work). In addition, morphologically small round cells gave rise to larger elongated cells which were not viable. While the lagging chromosome phenotype observed for the *cep2* mutants may occur as a result of the failure of the proteasome to degrade a substrate such as cyclin, which is required for the exit from mitosis, it is also possible that this phenotype, in the case of *cep2-10* and *cep2-13*, may result more from a defect in a putative helicase activity than from a defect in proteasomal function. While not preventing sister-chromatid segregation, a defect in a helicase might affect decondensation of the DNA or the association of the DNA with components of the mitotic spindle.

Sug1p has recently been shown to have 5'-3' DNA helicase activity. Mts2p could also be tested, as could the gene product of all of the alleles. An indication that this proteasomal ATPase has helicase activity, would necessitate a re-evaluation of the roles of the proteasomal ATPases in intracellular metabolism.

7.6 Future Work

There are many intriguing questions which arise as a result of the work presented here. One of these questions is an understanding of the function of the products of the *let1*⁺ and *aps1*⁺ genes. It is probable that Let1p and perhaps some of the other ATPase subunits are involved in binding to potential proteolytic substrates. This being so, one question which arises is whether the ATPase domains from one of the putative proteasomal ATPases could carry out the same function when fused to the non-

conserved N-terminal domain of another ATPase? This experiment would determine whether or not the ATPase domain performed a specific function, or whether the ATPase domain could bind proteins in a non-specific manner thereby leaving the N-terminus free to facilitate proteasomal targeting and assembly. This 'domain-swap' approach was used to identify functions associated with different regions of the *CDC34* (*UBC2*) and *RAD6* (*UBC3*) gene products from *Saccharomyces cerevisiae* (Kolman *et al.*, 1992; Silver *et al.*, 1992). Work is already underway to test this hypothesis. A positive result would imply that the N-terminus is specific for binding other subunits (ATPase and non-ATPase) during formation of the 26S proteasome whereas the CAD domains of all of the ATPases performed similar functions. The difficulty with this experiment lies in the inability to predict at which point to make the 'swap', but would perhaps best be attempted somewhere downstream of the non-conserved region. It is this region which varies most in length and primary structure.

Another relevant experiment is to show that binding of Let1p to Mts2p is in fact specific (see section 7.2). This can be done by tagging either Mts2p or Let1p with a 6-His epitope or haemagglutinin (HA) tag, expressing the recombinant proteins in *E.coli* and looking for specific association between the 2-proteins in an immunoprecipitation experiment. More specifically, it would be interesting to confirm the results of the 2-hybrid deletion analysis by investigating which of the deletions of the Let1 protein could still bind Mts2p. This technique has been used to demonstrate an *in vitro* association between Mts2p and Mts4p (Wilkinson *et al.*, 1997)

The isolation of *aps1*⁺ showed that this *MSS1/CIM5* homologue was present in fission yeast. Cloning of this cDNA under the control of the *nmt1* promoter in the pREP series of vectors will enable suppression studies to be done. It is predicted that this construct will rescue the *ts* phenotype of the *mts2-1* mutant, as was shown for *MSS1*, the murine homologue of *aps1*⁺ (Gordon *et al.*, 1993). However, it is possible that over-expression of *aps1*⁺ will also rescue some of the other *MBC*^R mutants. More specifically, the ability to rescue a mutation in an α -type subunit of the 20S proteasome would suggest that contact occurs between the 20S proteasome and the ATPase ring of the 19S regulatory complex. Although there is a report of a mutation in *SUG1* being able to suppress a mutation in a 20S subunit in budding yeast, this has not yet

been published nor demonstrated in any other organism. The production of antibodies against Aps1p will enable immunoprecipitation of complexes containing this protein, and, as was the case for Cdc48p in budding yeast (Ghislain *et al.*, 1996), might reveal some other unexpected complexes and functions for this molecule.

Having characterised a range of mutations in the *mts2* gene, it would be interesting to determine how these mutations might affect proteasomal assembly. Although all of them occur in the CAD domain of the molecule and might therefore be expected to have an effect on ATP hydrolysis or substrate binding, it is also possible that the mutants may affect incorporation of the mutant S4 subunit into the proteasome. So far, the 26S proteasome has only been purified from one allele of *mts2*. Analysis of these purified preparations has shown that the mutant S4 subunit is present in the proteasome, indicating that in this case, the 26S proteasome is not defective in assembly, but in *ts* conjugate degrading activity (Seeger *et al.*, 1996). Thus, it would be useful to perform similar experiments on purified proteasome preparations from the *mts2-16* and *mts2-25* mutants to assess the effect of these mutations on proteasome assembly and function.

Finally, given that no *ts* mutants in the *let1*⁺ or *aps1*⁺ genes have been isolated, it may be possible, using the information gained by analysis of mutations in the *mts2* gene, to create site-specific conditional mutations within the *let1* and *aps1* open reading frames. This would enable the isolation of suppressor mutations in each case, and may provide some more information about the specific functions of these proteasomal ATPases.

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APPENDIX A Oligonucleotides used during this work

Chapter3

3.3.1 Amplification of internal *mts2* fragment

C823 5' CGTTGGTTCTGTTCTTATTC 3' T_m=51°C

D858 5' CCTTTTCACGAGCTTGCCG 3' T_m=57°C

3.4.4.1 Sequencing of library plasmids from 2-hybrid screen

F444 5' TCTAGAACTAGTGGATC 3' T_m=43°C

F250 5' GTAAAACGACGGCCAG 3' T_m=47°C

3.4.4.2 Amplification of *let1* DNA sequence from library plasmids

F444 As above

I631 5' CTCCTTAATATCAATATCTGG 3' T_m=51°C

3.4.4.3 Amplification of insert from 2-hybrid library plasmids

F444 As above

F250 As above

3.4.4.4 Sequencing of the *S.pombe* homologue of MSS1

Forward

F444 As above

M159 5' GTTGCCAGATGTACCAAAATT 3' T_m=53°C

M213 5' CACTATGTGCTCGTGCTGTTG 3' T_m=59°C

M214 5' GAGAATTATTTGAAATGGCACG 3' T_m=55°C

M253 5' CCCTTCACAAACTGGTGCAGA 3' T_m=61°C

Reverse

F250 As above

M160 5' CTCCGCAGATTGTTTCATTCTC 3' T_m=57°C

M215 5' TGGCAACAGCACGAGCACATA 3' T_m=59°C

M254 5' GCACGAATCGCAAACATTCCA 3' T_m=57°C

Chapter4

4.2 Truncations of the Mts2p and Let1p open reading frames

J337 5' ATTCTCGAGATTTAGGCACGTGCC 3' T_m=60°C LCA1

J338 5' GTCCTCGAGACTTAGCTATCGCCA 3' T_m=57°C LCA2

J339 5' CTGCTCGAGCTTACAAGGTTT 3' T_m=51°C LCA3

J340 5' TAACTCGAGTCAGGAATTTATTCG 3' T_m=61°C LCA4

H789 5' TTAAGACCATGGGAAAGAACAAAGTTCTAGTCA T_m=48°C LNA1

K42 5' GTAGCCATGGGGAACGAT 3' T_m=51°C LNA2

K39 5' AGTCCCATGGTTCGTGAA 3' T_m=45°C LNA3

J341 5' CAACTCGAGTTCAGCGTTCAC 3' T_m=57°C MCA1

J342 5' CTTCTCGAGGATTCTAGAAAAGAA 3' T_m=57°C MCA2

J343 5' ACACTCGAGGGCTAATGTTCTTCG 3' T_m=63°C MCA3

J344 5' AAGCTCGAGAGCCTATTTGAT 3' T_m=53°C MCA4

J345 5' TACCTCGAGTTTAATCTACACGAT 3' T_m=61°C MCA5

Chapter 5

5.2 Cloning of *let1*⁺ into pSK⁺

J165	5' GCTCTAGAATCGATATAGAATTTTAACG 3'	Tm=45°C
J166	5' GGGGTACCATCGATTGGCTCTGGTTCGAAG 3'	Tm=63°C

5.2 Removal of *let1Δ::ura4⁺* from pSK-*let1Δ::ura4⁺*

I55	5' CGGAGGTGCTGAAGACTAACG 3'	Tm=61°C
K32	5' AACGAATAGTAAAGGATAGA 3'	Tm=47°C

Chapter 6

6.3 sequencing of the *mts2⁺* gene from MBC^R and *cep2* alleles

C699	5' TTCATCTATCGCTGAATAGT 3'	Tm=49°C
D757	5' GGAAGAAGAGTATGTGCAA 3'	Tm=53°C
D790	5' CGCAATATGTTAGAGTGCCTTG 3'	Tm=59°C
K61	5' GTACCATGGTTGAGTTACTC 3'	Tm=51°C
D858	5' CCTTTTCACGAGCTTGCCG 3'	Tm=55°C
C822	5' CATCTTGGAAGTGTGAATGG 3'	Tm=53°C
C657	5' ATTGCCATGGGATCATTGG 3'	Tm=51°C
J345	5' TACCTCGAGTTTAATCTACACGAT 3'	Tm=61°C